# ABSTRACT

# CHARACTERIZATION OF RHIZOSPHERIC ACTINOMYCETES OF MAJOR CROP PLANTS AND THEIR PLANT GROWTH PROMOTING PROPERTIES UNDER JHUM FIELDS OF MIZORAM

**A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**

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**DEPARTMENT OF FORESTRY**

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# CHARACTERIZATION OF RHIZOSPHERIC ACTINOMYCETES OF MAJOR CROP PLANTS AND THEIR PLANT GROWTH PROMOTING PROPERTIES UNDER JHUM FIELDS OF MIZORAM

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**IN PARTIAL FULLFILMENT OF THE DEGREE OF PHILOSOPHY IN FORESTRY OF MIZORAM UNIVERSITY**

**MIZORAM**

## DECLARATION

I Miss Marcy D. Momin**,** hereby declare that the subject matter of this thesis 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 do 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 the degree of Doctor of Philosophy in the Department of Forestry.

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## CERTIFICATE

This is to certify that the thesis entitled "**Characterization of rhizospheric actinomycetes from major crop plants and their plant growth promoting properties under jhum fields of Mizoram**" submitted to the Mizoram University, Aizawl for the award of the degree of Doctor of Philosophy in Forestry is the original work carried out by Miss Marcy D. Momin (Reg. No. MZU/Ph.D./1021 of 31.05.2017) under my supervision. I further certified that the thesis is the result of his own investigation and neither the thesis as a whole nor any part of it was submitted earlier to any University or Institute for the award of any degree. The candidate has fulfilled all the requirements laid down in the Ph.D. regulations of the Mizoram University.

His passion oriented hard work for the completion of the research is to be duly appreciated.

Date: Prof. S.K. Tripathi

Place: (Supervisor)

# TO WHOM IT MAY COMCERN

This is to certify that Miss Marcy D. Momin, a Ph.D Scholar, Registration No. MZU/Ph.D./1021 of 31.05.2017 has worked on the thesis entitled “Characterization of rhizospheric actinomycetes from major crop plants and their plant growth promoting properties under jhum fields of Mizoram”. He has fulfilled all criteria the mandatory publication. It is also certified that the scholar has been admitted in the department through an entrance test followed by an interview as per the regulation 2016.

Head

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**Place:** Aizawl, Mizoram **Marcy D. Momin**

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## List of abbreviations and symbols

| | |
|---|---|
| % | Percent |
| ºC | Degree Celsius |
| g | Gram |
| ml | Millilitre |
| m | Metre |
| mm | Millimeter |
| µm | Micrometer |
| µl | Microlitre |
| µg/ml | Microgram per millilitre |
| µg | Microgram |
| mM | Millilimeters |
| $cm^2$ | Centimeter Square |
| w/v | Weight/Volume |
| rpm | Revolutions per minute |
| O.D. | Optical Density |
| nm | Nanometer |
| Mins | Minutes |
| CFU | Colony Forming Unit |
| CAS | Chrome Azurol S |
| P | Phosphorus |
| K | Potassium |

| | |
|---|---|
| N | Nitrogen |
| SOC | Soil Organic Carbon |
| NaCl | Sodium Chloride |
| DMSO | Dimethyl sulfoxide |
| ISP2 | International StreptomycesProject 2 |
| ISP1 | International Streptomyces Project 1 |
| IAA | Indole-3-acetic acid |
| SCA | Starch Casein Agar |
| CSM | Cross Streak Media |
| L-Tryptophan | Levorotatory form of tryptophan |
| $KNO_3$ | Potassium nitrate |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| $MgSO_4.7H_2O$ | Magnesium sulphate |
| $CaCO_3$ | Calcium Carbonate |
| $FeSO_4.7H_2O$ | Ferrous sulfate heptahydrate |
| $K_2HPO_4$ | Dipotassium phosphate |
| $FeCl_3$ | Iron(III) chloride |
| $HClO_4$ | Perchloric acid |
| $Ca_3 (PO4)_2$ | Calcium phosphate |
| Fe | Iron |
| Zn | Zinc |
| FePO4 | Iron(III) phosphate |
| K-solubilizing bacteria | Potassium solubilizing bacteria |

| | |
|---|---|
| T | Treatment |
| C | Control |
| ANOVA | Analysis of Variance |
| LSD | Least Significant Difference |
| SA | Surface Area |
| AD | Average Diameter |
| AL | Average Length |
| FW | Fresh Weight |
| DW | Dry Weight |
| SE | Standard Error |
| AM | Aerial Mycelium |
| SM | Substrate Mycelium |
| PGP | Plant growth-promoting |
| PGPR | Plant growth-promoting rhgizobacteria |
| KVK | Krishi Vigyan Kendra |
| DNA | Deoxyribonucleic acid |
| 16S rRNA | 16S ribosomal RNA |
| PCR | Polymerase Chain Reaction |
| Tag-polymerase | Thermus aquaticus Polymerase |
| MEGA | Molecular Evolutionary Genetics Analysis |
| Blast N | Basic Local Alignment Search Tool for Nucleotides |

| | |
|---|---|
| NCBI | National Center for Biotechnology Information |

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# Chapter 1

# Introduction

## 1.1. The rhizosphere: structure and function

The rhizosphere, a narrow region of the soil around roots, is influenced by root secretions and numerous associated soil microorganisms. The roots exude an array of carbon containing compounds and add dead cells and slough in the rhizosphere which is known as rhizodeposition. The process of rhizodeposition plays an important role in defining inhabitant microbiota, which differs from that of the bulk soil. The rhizosphere is considered as one of the most complex ecosystems on the Earth as result of dynamic association of microbial community. This region is rich in variety of carbon compounds exuded from plant roots which provide unique environments for assemblage of diverse soil microorganisms including bacteria, fungi, actinomycetes, archaea, oomycetes, nematodes, protozoa, algae, viruses, archaea, and miro-arthropods. The microbial communities in the rhizosphere are distinct from the microbial communities of bulk soil (Mendes *et al*., 2013). Rhizosphere represents biological niche for diverse saprophytic micro-organisms because of high input of organic matter derived from plant roots and root exudates (Merckx *et al*., 1987). Microbial community structure in the rhizosphere is affected by plant composition, soil types and the environments which further affect the interactions between microorganisms and soil and plants (Dey *et al*., 2012).

Rhizosphere possesses is strongly affected by various carbon compounds (i.e. sugar compound, amino acid, enzyme, vitamin, organic acid, nucleotide, antibiotic, phenolic compound, or other types of compounds) secreted by plant roots (Rovira, 1969). Variation in the composition of root exudates is determined by the type of plant, growth phase, and physical factor like pH, type of soil, humidity, temperature, and the presence of microorganisms (Huang *et al*., 2014). The composition of exudates alters the microflora and ultimately chemical structure of the rhizosphere in comparison to the bulk soil. Exudates derived in the form of chemicals released into the rhizosphere by cells in the roots and cell waste referred as "rhizodeposition",

serves as source of energy for the rhizospheric microorganisms. Structure and constituents of the plant exudates can be varied including sugars, lipids, and proteins (high molecular weight) and organic and amino acids (low molecular weight). Plant exudates structure and constituent can be often plant-species-specific (Badri and Vivanco, 2009). Sugars and amino acids comprise most plant root exudates and they help in a variety of functions including as antimicrobials, allelopathic molecules, and pathogen/herbivore defences. Outside the rhizosphere area, it is prominent at some plants secrete allelochemicals (e.g. flavanols, carbohydrates and phenols) from their roots which inhibit the growth of other organisms. Numerous rhizosphere inhabitant microorganisms play vital roles in ecological fitness of the plant host. The rhizosphere microbiome affects plant growth, development, biotic and abiotic stress resistance through altering the absorption of nutrients into plant cells, the exchange of chemical signals, and affects enzyme activity during metabolic processes (Lugtenberg and Kamilova, 2009; Berendsen *et al*., 2012; Soussi *et al*., 2015; Lareen *et al*., 2016; Qiao *et al*., 2017). Rhizosphere organisms that are harmful to plant growth and health include the pathogenic fungi, oomycetes, bacteria, and nematodes.

Plant-microbe interactions may thus be considered beneficial, neutral, or harmful to the plants depending on the specific microorganisms, plants and the prevailing environmental conditions (Bais *et al.,* 2006). Rhizospheric microbes are not only results in high plant-growth promoting properties on the root systems but it also functions as biocontrol microorganisms which are involved in direct inhibition of plant pathogens (Shoda, 2000; Raaijmakers *et al*., 2002). It includes antibiosis i.e. the inhibition of microbial growth by diffusible antibiotics and volatile organic compounds, toxins, and biosurfactants, and parasitism that may involve production of extracellular cell wall-degrading enzymes such as chitinases and β-1, 3-glucanase (Compant *et al*., 2005; Haas *et al*., 2005). The quantity of species of bacteria including actinomycetes in the rhizosphere can vary from thousands to millions per gram. The presence of actinomycetes in plant rhizospheres has specificity founded on the strain and plant species (Rovira, 1969; Intra *et al.*, 2011).

## 1.2. Characteristics of actinomycetes

Actinomycetes are unicellular gram-positive bacteria showing a filamentous growth like fungi, and are ubiquitous in nature showing wide distribution in a variety of natural ecosystems around the world (Chamikara, 2016). They are aerobic and predominant in dry alkaline soil (Jeffrey, 2008). Number of studies have reported actinomycetes from various ecosystems including terrestrial soils (Jeffrey, 2008; Salim *et al*., 2017), marine ecosystem (Attimarad *et al*., 2012; Mohseni *et al*, 2013), mangrove ecosystem (Mangamuri *et al*., 2012; Abidin *et al*., 2016), composts (Workie and Abate, 2016) and vermicomposts (Gopalkrishnan *et al*., 2011). Actinomycetes are rich in G+C (guanine and cytosine) content with 57-75% GC in their DNA (Lo *et al*., 2002). These organisms with characteristics common to both bacteria and fungi but possessing distinctive features to delimit them into a distinct category. They morphologically resemble fungi and physiologically bacteria (Sultan *et al*., 2002).

Actinomycetes are thread-like filaments in the soil and grow as hyphae like fungi responsible for the characteristically earthy smell of freshly turned healthy soil. Presence of actinomycetes can potentially serve as an indicator of compost maturity and they also participate in suppressing of pathogens in the curing stage (Steger *et al*., 2007; Tang *et al*., 2006) which is accompanied by the presence of pleasant earthy smell due to release of a chemical geosmin by them (Wilkins, 1996). Actinomycetes are characterized by the formation of threads, filaments, or strands, which spread throughout as compost heap or soil. Actinomycetes form normally branching threads or rods. The hyphae are generally non-septate. The sporulating mycelium was found aerial and substrate; and may be branching or non-branching, straight or spiral shaped. The spores are spherical, cylindrical or oval (Chamikara, 2016). Due to their filamentous nature and cultural characteristics they have been placed within the phylum Actinobacteria, class Actinobacteria, sub-class Actinobacteridae, order Actinomycetales which currently consists of 10 sub-orders, more than 30 families and over 160 genera (Chavan *et al*., 2013).

Actinomycetes on culture media show different cultural characteristics when grown on agar surface. International Streptomyces Project (ISP) has suggested different

media to distinguish and characterize actinomycetes at different levels (Shirling and Gottlieb, 1976). Number of researchers have used isolated actinomycetes from soil and other natural habitats using different media such as starch casein agar [SCA] (Kuster and Williams, 1964, Duddu *et al*., 2016), actinomycete isolation agar [AIA] (Pattnaik and Reddy, 2012; Pandey *et al*., 2011), glycerol asparagine agar [GAA] (Pridham and Lyons, 1961; Low *et al*., 2015), Oatmeal Agar [OMA] (Low *et al*., 2015), yeast malt extract Agar [ISP2] (Mohseni *et al*., 2013). Actinomycetes branch and form network of hyphae growing on both the medium, for example, on the surface of agar (aerial mycelium) and under the surface of agar (substrate mycelium). Their growth is considered by small compact, soft, hard, sticky, gel-like colonies persistently adhering to the medium, the surface being flat, convex, raised, umbonate and crateriform (Sathi *et al*., 2001).

The outer zones of the colonies are smooth but fringes of minute hyphae are observed under the low power microscope (Muiru *et al*., 2008). The colour of the aerial mycelium can vary from white, creamy white, chalky, orange, dark-pink, powdery, brown, grey to pinkish and violet and substrate mycelium may vary from brown, yellow to orange (Mohseni *et al*., 2013; Jeffrey, 2008; Salim *et al*., 2017; Amit, 2011). They are also form concentric rings on aging (Sathi *et al*., 2001). Many cultures on the surface appears almost same but they are found different characteristics when observed from the reverse side of the culture petri-plate, this may be due to differences in substrate hyphae. They are also found producing pigments vary from blackish brown, yellow to orange, brown orange, brown red (Shirling and Gottlieb, 1966; Mohseni, *et al*., 2013). Many cultural characteristics of actinomycetes have been employed for the determination of their classification such as actinomycetes are grown on tyrosine agar (ISP7) show melanin pigment production (Pridham *et al*., 1957; Duddu *et al*., 2016), ability to utilize various carbon sources for energy which is determined by their growth on carbon utilization medium [ISP 9] (Pridham and Gottlieb, 1948). The most dominant discovered actinomycetes from soil are those belonging to the *Streptomyces* genus. Lihua *et al*. (1996) reported *Streptomyces* to be the most important genus in ecological function. *Streptomyces* are an economically important group of organisms among

actinobacteria family. They are responsible for the production of about half of the discovered metabolites, notably antibiotics, antitumor agent, an immunosuppressive agent, enzymes and enzymes inhibitors.

### 1.3. Functions of actinomycetes in the rhizosphere

Actinomycetes are active in the plant rhizosphere and help in the degradation of a wide range of biopolymers by secreting several hydrolytic enzymes and tolerate hostile conditions by forming spores (Alexander, 1977). Some actinomycetes secrete a range of enzymes that can completely degrade all the components of lignocelluloses such as lignin, hemicellulose and cellulose (Limaye *et al*., 2017). Actinomycetes secrete amylases in the outside of the cells to carry out extracellular digestion of amylase starch degrading amylolytic enzymes which has application in biotechnological applications of food industry, fermentation, textile and paper industries (Pandey *et al*., 2000). The production of cellulases by actinobacteria which are a collection of hydrolytic enzymes that hydrolyze the glucosidic bonds of cellulose and related cello-digosaccharide derivatives (Ito, 1997). In the current industrial processes, cellulolytic enzymes are employed in the color extraction from juices, detergents causing color brightening and softening, biostoning of jeans etc. (Zhou *et al*., 2001). *Streptomycetes* soil inhabitant microorganism has been discovered recently which has importance for their complex interactions with plants and other organisms (Seipke *et al*., 2011).

Apart from saprophytes, actinomycetes have been continuously reported as most prolific producers of microbial bioactive secondary metabolites for potential agricultural, pharmaceutical and industrial applications (Gesheva and Gesheva, 2000; Balachandran *et al*., 2012; Dasari *et al*., 2012; Abidin *et al*., 2016) such as antibiotics, enzymes, antitumors agents, biopesticises, plant growth promoting hormones. Among this group of bacteria, 7600 (76%) compounds are reported from a single genus, *Streptomyces* (Berdy, 2012). The most striking fact is that these filamentous bacteria have evolved with the wealth of biosynthetic gene clusters and thereby show an unprecedented potential in production of biologically active natural product scaffolds (Jose and Jha, 2016). Actinomycetes are present extensively in the plant rhizosphere and produce various agro-active compounds (Anwar *et al*., 2016).

In the previous research due to its soil dominant saprophytic nature, this group of bacteria added prime attention as plant growth promoters (Franco-Correa *et al*., 2010).

In addition, the rhizosphere actinomycetes possess potential of PGP properties in the rhizosphere either directly or indirectly, and thereby they have been reported to increase crop productivity. According to Sharma (2014), most agriculturally important actinomycetous genera have been exploited only from two families (i.e. Actinomycetaceae and Streptomycetaceae). PGP actinomycetes improves the growth and vigour of plant by producing phytohormones like indole acetic acid (IAA), cytokinins, and gibberellins (Marques *et al*., 2010); solubilizing inorganic phosphate (Jeon *et al*., 2003); fixing asymbiotic nitrogen (Khan, 2005), producing siderophores, antibiotics and fungicidal compounds which are responsible for antagonistic effect against phytopathogenic microorganisms (Lucy *et al*., 2004; Barriuso *et al*., 2008; Majeed *et al*., 2015).

Further, strains of *Streptomyces* sp. have been reported to show maximum phosphate solubilization activity (Verma *et al*., 2001). Almost all the PGP actinomycetes were able to synthesize IAA which stimulates adventitious roots that help plant to absorb nutrients and water from a large volume of soil along with increased amount root exudates which in turn promotes bacterial association (El-Tarabily, 2008). Siderophore production stimulates plant growth by forming complex iron form (Fe 3+) in the rhizosphere making iron unavailable to the phytopathogens and promoting growth of the plant (Tan *et al*., 2009). Majority of the rhizospheric actinomycetes synthesize ammonia and supply nitrogen to the host plant. Ammonia production helps in HCN production which serves as a factor for prompting plant disease suppression (Marques *et al*., 2010). The association of actinomycetes with plant rhizosphere benefits the growth of plants by producing phytohormones, increasing nutrient uptake of plants and inhibiting the growth of pathogens by producing various antipathogenic compounds such as siderophores, β-1, 3-glucanase, chitinase, antibiotics, and cyanide (Shimizu, 2011).

## 1.4. Rhizospheric soil characteristics

The properties of the rhizosphere soil are recognized to be influenced by the presence and the activity of plant roots and their associate microbes which causes acidification through proton extrusion and the release of root exudates (Grayston *et al*., 1996; Jones *et al*., 2009). Plant rhizosphere soil embodies a biological position with a diverse microflora comprised of bacteria, fungi, protozoa, and the algae. This community is maintained nutritionally by a high input of organic material resulting from the plant roots and root exudates that are required for microbial growth (Panhwar *et al*., 2012). Rhizosphere soil is nutritionally rich niche providing different growth factors to the associated microflora. Isolation of various groups of actinomycetes from the rhizosphere of different plant species have been reported previously (Zhong *et al*., 2011). Actinomycetes are abundantly distributed in the rhizosphere and colonized plant roots which play an important role in plant growth promotion (Chaiharn *et al*., 2018). Actinomycetes can adopt to survive for a long time in the various types of soils due to their spore forming ability, and are capable of producing various bioactive compounds such as antibiotics, siderophores, chitinase, phytohormones along with phosphate solubilizers (Navon, 2000). Environmental factors affect the type and population of actinomycetes in the soil. They have been reported to live in both mesophillic (25- 30 C) and thermophillic (40ºC) environments (Haseena *et al*., 2016). The pH of the soil is also a major environmental factor determining the distribution and activity of actinomycetes. Most of the actinomycetes grow at optimum pH around 7. Vasavada *et al*. (2006) showed that pH, salinity, use of media and carbon and nitrogen sources affect the growth and antibiotic production of actinomycetes. Most of the mesophillic actinomycetes are active in compost in the initial stages of decomposition. However, the capacity of self-heating during decomposition provides ideal conditions for thermophillic actinomycetes (Chavan *et al*., 2013). Plant species, plant developmental stage and soil type have been indicated as major factors determining the composition of rhizosphere microbial communities (Broeckling *et al*., 2008).

## 1.5. Soil fertility in shifting cultivation and actinomycetes

Shifting cultivation is dominant agriculture practice in the Northeast, India. This system involves selection of fields in the months of December and January, cutting and clearing of vegetation, drying and burning of herbs, shrubs, twigs and branches in the month of February and March for the cultivation of crops followed by seeding in the months of April and May. Generally, mixed crops are grown in this system of cultivation. Rice is the major food crop grown in shifting cultivation followed by maize, French bean, yam, chilly, brinjal, cucumber, pumpkin, bitter guard, tapioca, squash, bottle gourd, cow pea, tomato and flat bean under (Sati and Rinawma, 2014). Rice (*Oryza sativa* indica L.), maize (*Zea mays* L.), garden pea (*Pisum sativum* L.), yam (*Dioscorea* sp. L.), bird's eye chilli (*Capsicum annuum*), eggplant (*Solanum melongena*), Ethipian eggplant (*Solanum aethiopicum*) are the major crops commodities widely cultivated in Mizoram, Northeast, India. The farmers grow crops for few years depending on the levels of soil fertility and left the land abandoned as fallow to recover the soil fertility through natural plant regeneration. Previously, the fallow length (e.g. 20-25 years) was sufficient allow the system to fully recover, and the system was fairly productive and ecologically balanced. However, in the recent years the fallow length has been significantly decreased to <5 years due to increasing population pressure (Grogan *et al*., 2012). Since different plants produce different chemical metabolite, so in order to survive the microbes' actinomycetes need to adapt to the environment (Oskay *et al*., 2004). Actinomycetes diversity can also be influenced by the array of plant species grown on shifting cultivation soil. As there is variation in the microbial diversity under different soil types with different host plant, the present study aimed to isolate and characterized potential PGP rhizospheric actinomycetes of major crop plants under shifting cultivation of Mizoram of the Northeast, India.

## 1.6. North-Eastern (NE) region

The North Eastern (NE) region of the India, comprising eight states (e.g. Arunachal Pradesh, Assam, Meghalaya, Manipur, Mizoram, Nagaland, Tripura, Sikkim) are the part of Indo-Burma biodiversity hotspot which represents unique habitat for the enormous amount of flora and fauna which are under threat due anthropogenic

activity. The region is the geographic entry for abundant of India's diversity of the living organisms (Tripathi *et al*., 2016). Amongst all the states, Mizoram have been reported highest percentage of forest cover with a characteristic of steep slopes Mizoram covers an area of 21,087 $km^2$ and about 91 percentage of the state is forested (SFR, 2011). Therefore, existence of agriculturally and industrially potential actinomycetes strains with diverse genetic resources cannot be ruled out. It is well recognized that the diversity of microbial community especially rhizospheric actinomycetes in these region remains unidentified and uncharacterized. Moreover, report of studies on the genetic diversity of rhizospheric actinomycetes and their plant growth promotion are scanty in this region.

Therefore, it is important to study and conserve the genetic diversity along with potential plant growth promoting properties of rhizospheric actinomycetes associated with major crop plants under shifting cultivation of this region. Further, plant growth promoting properties will allow us to understand the role of rhizospheric actinomycetes and their application in enhancing plant growth in shifting cultivation. Present study presumed that actinomycetes may have huge impact on rhizosphere of the major crops and influence soil properties which in turn increase crop productivity and soil health. The present study is designed to obtain the potential isolates of actinomycetes from the rhizosphere of crops, which have the ability in indole, ammonia, siderophore production and phosphate solubilization necessary for crop production and yield.

### 1.7. Objectives are:

1) T o isolate and characterize actinomycetes from the rhizosphere of major crop plants of Mizoram.

2) To screen out identified isolates of actinomycetes for their plant growth promoting properties.

3) To determine physico-chemical properties of different plant rhizosphere soils and to relate plant growth promoting (PGP) properties of actinomycetes with rhizosphere soil characteristics.

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# Chapter2

## Review of Literature

### 2.1. Microorganisms and their applications

Microorganisms are diverse group of microscopic organisms including bacteria, fungi, actinomycetes, archaea, protozoans and some algae, which are ubiquitous in nature and found in diverse habitats for examples, soil, hot springs, up to "7 miles deep" in the ocean, up to "40 miles high" in the atmosphere and inside rocks of deep Earth's crust (Bhattarai *et al*., 2015). Microorganisms are exploited for both traditional food and beverage preparation using modern biotechnological technologies based on genetic engineering (Bourgaize *et al*., 2004), and are important source of natural compounds of agro active importance. In recent years, application of microbial consortia in the form of bio fertilizers is used for the reduction of chemical fertilizers, pesticides and related agrochemicals without compromising the plant yield (Ahmad *et al*., 2008). Therefore, increasing understanding of indigenous actinomycetes from crop plants of shifting cultivation would be important to formulate biofertilizer in traditional agricultural practices in Mizoram, northeast.

### 2.2. General characteristics of actinomycetes

Actinomycetes are ubiquitous in nature and exhibit a unique metabolic diversity and enzymatic capabilities by producing the secondary metabolites which are valuable for agricultural, industrial and pharmaceutical purposes. A variety of actinomycetes inhabit wide range of plants either as symbionts or endophytes (Matsukuma *et al*., 1994; Okazaki *et al*., 1995). Endophytic actinomycetes are now recognized as potential source of novel antibiotics and physiological activators (Igarashi *et al*., 2000). A number of actinomycetes isolated from soil and rhizosphere has been recommended for the biocontrol of fungal root and seed pathogens (Felsenstein, 1993; Cresswell *et al*., 1992: Distler *et al*., 1992: Kortemaa *et al*., 1994; Tahoven *et al*., 1995). Actinomycetes are also known as prolific producers of commercially important clinical antibiotics (Okami and Hotta, 1988). Actinomycetes are a group of

prokaryotic organisms belonging to subdivision of the Gram-positive bacteria phylum. Most of them are in sub-class -Actinobacteridae, order- Actinomycetales. The members of this order are characterized in part by high G+C content (>55 mol %) in their DNA (Stackbrandt *et al*., 1997), and are filamentous bacteria which produce two kinds of branching mycelium, aerial mycelium and substrate mycelium. The aerial mycelium is important part of the organism that produces spores, and thus they have been considered as fungi, as reflected by their name, akitino means ray and mykes means mushroom/fungus, so actinomycetes was called ray fungi. Actinomycetes are the most widely distributed group of microorganisms in nature and are also well known saprophytic soil inhabitants (Takizawa *et al*., 1993). The soil actinomycetes produce a volatile compound called geosmin, which literally translates the "earth smell" (Gust *et al*., 2003). This organic compound is responsible for a contributor to the strong odour that occurs in the air when rain falls after a dry spell of weather. In natural habitats, *Streptomyces* are common and are usually a major component of the total actinomycetes population. Some genera of actinomycetes i.e. *Actinoplanes, Amycolatopsis, Catenuloplanes, Dactylosporangium*, *Kineospora*, *Microbispora*, *Micromonospora*, *Nonomuraea* are often very difficult to isolate and cultivate due to their slow growth are called rare actinomycetes (Hayakawa, 2008).

### 2.3. Isolation of actinomycetes from soil

Members of actinomycetes especially *Streptomyces* and *Micromonospora* have long been recognized as major producers of useful natural secondary metabolites (Miyadoh, 1993). However, the rate of discovery of new metabolites from these common actinomycetes has declined. Thus, the selection of improved methodologies for isolating the uncommon and rare actinomycetes is required to avoid the re-isolating strains that produce known bioactive metabolites and to improve the quality of the natural products screened (Takahashi and Omura, 2003; Berdy, 2005). Various media and methods including the techniques that enhance the desirable actinomycetes in natural habitat samples (enrichment) or eliminate undesirable *Streptomyces* and other contaminants from the isolation plate media (pretreatment)

for isolating novel actinomycetes from natural habitats, especially from various types of soil, were improved and developed.

### 2.4. Identification of actinomycetes

Morphological observations including germination of spores, elongation and branching of vegetative mycelium, formation of aerial mycelium, color of aerial and substrate mycelium and pigment production have been used to identify actinomycetes (Holt *et al*., 1994). Formation of aerial mycelium, substrate mycelium and spores were studied by light microscopy and the spore surface and spore structure by scanning electron microscopy. Based on the aforementioned characteristics, genus level identification of the potential strain was made by Bergey's Manual of Systematic Bacteriology (Locci, 1989).

At present, the molecular biological identification is based on 16S rDNA sequences which are the most significance for actinomycetes identification (Yokota, 1997). The phylogenetic tree constructed from 16S rDNA sequences allows the investigation of actinomycetes evolution. Based on the 16S rRNA gene sequencing, actinomycetes are separated into over 100 genera. Molecular biological techniques have helped on large scale in finding new antibiotics from actinomycetes. With the advancement of technology in molecular study, primers had been developed by researchers to target specifically the 16SrRNA sequence of the actinomycetes (Schwieger and Tebbe, 1998; Wang *et al*., 1999). Identification of actinomycetes to genus level has been a great advancement in the area of identification as that the ability to obtain the genus of the actinomycetes in just a few hours is now possible. Exploring the biology of secondary metabolites production in actinomycetes through genetics has provided a foremost share to our current knowledge. As a noteworthy foundation, *Streptomyces* coelicolorA3 has genetically been recognized as a model for the actinomycetes, and the whole genome was announced with versatile in vivo and in vitro genetics (Bentley *et al*., 2002; Jose and Jha, 2016). Improvements made in bioinformatics methods, particularly specific for natural product gene cluster identification and functional prediction aids in the processing of bulk genomic data of actinomycetes (Alam *et al*., 2011; Doroghazi *et al*., 2014; Abdelmohsen *et al*., 2015).

## 2.5. Plant growth promoters

Plant growth promoting rhizobacteria (PGPR) is a group of naturally occurring, free living rhizosphere colonizing bacteria that improve plant growth, increase yield, enhance soil fertility, and reduce pathogens as well as biotic or abiotic stresses (Vessey, 2003; Kumar *et al*., 2015). PGPR help the plants by producing plant growth phytohormones such as indole acetic acid (IAA), cytokinins, and gibberellins (Marques *et al*., 2010), solubilization of inorganic phosphate (Jeon *et al*., 2003), asymbiotic nitrogen fixation (Khan, 2005), antagonistic effect against phytopathogenic microorganisms by producing siderophore, antibiotics, and fungicidal compounds (Lucy *et al*., 2004; Barriuso *et al*., 2008; Majeed *et al*., 2015). Some actinomycetes have ability to produce plant growth promoter substances which are important for plant growth and can apply for agriculture. Soil actinomyctes, especially *Streptomyces* represent an important source of biologically active compounds with high commercial value and important applications in human and livestock medicine and agriculture (Watve *et al*., 2001; Berdy, 2005). The biological active compounds produced by actinomycetes are antibiotics, immunosuppressant, extracellular hydrolytic enzymes, plant growth promoters and siderophores. Several researches reported that actinomycetes can produce auxins, gibberellins and cytokinins (El- Tarabily and Sivasithamparam, 2006).

### 2.5.1. Indole acetic acid (IAA)

Indole acetic acid (IAA) is a common natural auxin and is a product of L-tryptophan metabolism in microorganisms. Auxins are a group of indole ring compounds which have the ability to improve plant growth by stimulating cell elongation, root initiation, seed germination and seedling growth (El- Tarabily, 2008). Approximately 80% of rhizosphere bacteria can secrete IAA (Bhavdish *et al*., 2003). *Streptomyces* sp., inhabiting the rhizospheres of various plants, also serves as good source of IAA. Generally microorganisms isolated from the rhizosphere and rhizoplane of various crops have more potential of producing auxins than those from the root free soil (Arshad and Frakenberger, 1998).In the rhizosphere soils, root exudates are the natural source of tryptophan for rhizosphere micro-organisms, which may enhance auxin biosynthesis in the rhizosphere. Several Streptomyces species such as

*Streptomyces olivaceoviridis, Streptomyces rimosus and Streptomyces rochei* were isolated from the tomato rhizosphere, have the ability to produce IAA and improve plant growth by increased seed germination, root elongation and root dry weight (Aldesuquy *et al*., 1998; Tokala *et al*., 2002 and El-Tarabily, 2008).

Nowadays, some rhizospheric actinomycetes are studied and developed as a commercial product. For example, Mycostop, based on strain K61 of *Streptomyces grieseoviridis and Streptomyces lydicus* WYEC 108 can produce IAA to promote plant growth (Mahadevan and Crawford 1997). It was found by El-Tarabily and Sivasithamparam (2006) and Tsavkelova *et al*. (2006) that *Streptomyces* from many crop rhizosphere soils have the ability to produce IAA and promoted plant growth. It is possible that high tryptophan will be present in root exudates of lemongrass and enhance IAA biosynthesis in *Streptomyces* CMUH009. Rhizosphere soils of medicinal plants may be attractive sources of *Streptomyces* sp. capable of producing bioactive compounds related to plant growth promotion (Thangapandian *et al*., 2007).

### 2.5.2. Phosphate solubilization

Phosphate-solubilizing bacteria (PSB) have been reported in majority of soils (Chonker and Tarafdar, 1984; Venkateswarlu *et al*., 1984). Phosphorus is one of the most important nutrients for plant growth. Its major physiological role being in certain essential steps in accumulation and release of energy during cellular metabolism (Alexander, 1977). Phosphorus in soils is immobilized or becomes less soluble either by adsorption, chemical precipitation or both. Phosphorus availability to crops is subjected to chemical fixation in soil with other metal cations, depending on soil pH. A large number of microorganisms including bacteria, fungi and actinomycetes are known to produce acidic metabolites which by change of soil pH or by direct chelation of metal cations, release fixed or insoluble phosphorus in available form (Storkanova *et al*., 1999, Narsian and Patel, 2000, Reyes *et al*., 2002). Many species of actinomycetes are able to solubilise phosphates in vitro and most of them live in the plant rhizosphere.

Actinobacteria isolated from the rhizosphere was capable of increasing availability of phosphorus to plants either by mineralization of organic phosphate or by solubilisation of rock phosphate by production of acids (Hinsinger *et al*., 2003). Among the six selected actinomycetes, *Streptomyces griseus, Streptomyces cavourensis, Micromonospora aurantiaca strains* show significantly improved wheat plant growth in test tubes as well as in rock phosphate amended soil. The strains showing the best phosphate release abilities were also having most important stimulatory effect on shoot and root growth of the plant. (Hamdali *et al*., 2008).

### 2.5.3. Siderophores

Siderophores are the compounds that have ability to bind Fe3+, transport it back to the microbial cell and make it available for growth. The soil actinomycetes, especially species of Streptomyces, have been reported to produce siderophores (Tokala *et al*., 2002). Siderophores were used for agriculture and medical treatment. Microbial siderophores may also be utilized by plants as an iron source (Bar-Ness *et al*., 1991; Wang *et al*., 1993). Rhizosphere soil actinomycetes have to compete with other rhizosphere bacteria and fungi for iron supply and therefore siderophore production may be very important for their growth. Competition for iron is also a possible mechanism to control the phytopathogens in agriculture. Actinomycetes produce these compounds for compete iron with plant pathogenic fungi (Muller *et al*., 1984; Muller and Raymond, 1984). Nowadays, siderophores from actinomycetes were used for clinical application. The siderophores, desferrioxamine, from *Streptomyces pilosus* and oxachelin, from *Streptomyces* sp. GW9/1258 were used for treatment of iron overload and removal of other toxic metal from human tissue (Neilands, 1995).

*Streptomyces lydicus* WYEC108 originally isolated from a rhizosphere soil of linseed was also found to produce hydroxamate type siderophore (Hamby, 2001) converts nitrate to nitrite and ammonia. Nitrate reducing strains were enumerated from the rhizosphere of Glyceria maxima that ranged from 3.2 x 106 to 3.3x 108cfu g- 1(Nijburg *et al*., 1997) and they have also found that the total number of potential nitrate reducing strains in the rhizosphere significantly increased with NO3- addition. It was reported by El-Tarabily and Sivasithamparam (2006) that isolates of

*Microbispora rosea*, *Micromonospora chalcea* and *Actinoplanes philippinensis* produced X-1, 3, X-1, 4 and X-1, 6-glucanases, caused lysis of *Phytophthora aphanidermatum* hyphae in vitro and reduced.

### 2.5.4. Nitrogen fixation

Nitrogen is essential to all living organisms. Although 78% of the atmosphere consists of dinitrogen, nitrogen in this form cannot be used by most organisms and consequently the availability of nitrogen in a form suitable for assimilation is often a major limiting factor for growth. Biological nitrogen fixation is a process of reduction of atmospheric nitrogen to ammonia by free-living or symbiotic bacteria possessing the enzyme nitrogenase. Biological nitrogen fixation is of tremendous importance to the environment and to world agriculture. This process is an important part of the nitrogen cycle as it replenishes the overall nitrogen content of the biosphere and compensates for the losses that are incurred owing to denitrification. The increased use of chemical fertilisers, which constitutes the largest human interference in the nitrogen cycle, has prompted concerns regarding the increased emissions of nitrogen oxides, soil acidification and water eutrophication. The fixed nitrogen that is provided by biological nitrogen fixation is less prone to leaching and volatilization as it is utilized in situ and therefore the biological process contributes an important and sustainable input into agriculture (Dixon and Kahn, 2004). Some 23 genera of woody plants in 8 families are capable of forming symbiotic N2 –fixing associations with soil actinomycetes of the genus Frankia (Bond, 1976). Frankia are nitrogen-fixing actinomycete symbionts that cause the formation of perennial nodules on the roots of a botanically diverse group of plants. The association is referred to as “actinorhizal” (Lechevalier, 1994). These actinorhizal species are important components of many natural ecosystems and some may fix N2 at rates comparable to agricultural legumes (Torrey, 1978).Two strains, designated D ll and G2, were isolated from root nodules of *Casuarina equisetifolia*. This was the first report of nitrogen fixation by free-living actinomycetes isolated from nodules of a nitrogen-fixing non-legume. Acetylene reduction was ca. 10 to 30 nmol/h per mg of protein which is comparable to values obtained for free living Rhizobium strains which exhibited moderate acetylene reduction activities. *Streptomyces*

*thermoautotrophicus* UBT1 fixes N2 based on 15N analysis and growth was also observed in N-free medium (Gauthier *et al*., 1981).

Nitrogen fixation by symbiotic associations between soil bacteria belonging to the actinomycetes and root systems of a diversified group of woody dicotyledonous plants is less generally well known than that by the legume-Rhizobium symbiosis. The actinomycete induced nodulation of plants like the Alders (*Alnus*), bog plants like sweet gale (*Myrica gale*), sweet fern (Comptonia), bayberry (*Myrica pensylvanicum*) and various species of Ceanothus and their role in $N_2$ fixation have begun to be recognized as one of the largest sources for biological fixation of atmospheric dinitrogen. Even the ability of Frankia isolates to fix nitrogen is not apparently unique but may be shared by certain *Streptomyces* (Gadkari *et al*., 1992). At present, about 160 species in 15 genera among 7 families have been reported worldwide to have actinomycete induced nodulated nitrogen fixation. Furthermore, few of these plants have been of direct agricultural importance in the commerce of man (Torrey, 1978).

### 2.5.5. Enzyme production

The genus *Streptomyces* were shown to exhibit high alpha amylase production. Kumar *et al*. (2012) in their study screened actinomycetes from earthworm castings for their antimicrobial activity and industrial enzymes. Actinobacteria are important microorganisms that produce various useful enzymes and secondary metabolites such as immunomodulators, antitumor compounds and antibiotics (Saadoun *et al*., 2015).

## 2.6. Rhizosphere actinomycetes

The characteristics of the rhizosphere microbiome have previously been reported for some crops, such as rice (Edward *et al*., 2015), corn (Li *et al*., 2014; De la Cruz-Barron, M. *et al*., 2017), wheat (Donn *et al*., 2015), and sweet potato (Marques, J. M. *et al*., 2014). Sujatha, (2018) has been isolated antagonistic actinomycetes species from rhizosphere of cotton crop. (Qiao *et al*., 2017) were studied variation in the rhizosphere microbiome of cotton with soil type, genotype and developmental stage. Actinobacteria, especially *Streptomyces*, also exhibit immense biocontrol action against a range of phytopathogens (Wang *et al*., 2013). *Streptomycetes* have been

long considered simply as free-living soil inhabitants, but recently the importance of their complex interactions with plants, and other organisms is being uncovered (Seipke *et al*., 2011). The *Streptomyces* strains are extensively reported in the literature for its PGP potential (Nassar *et al*., 2003; El-Tarabily, 2008; Gopalakrishnan *et al*., 2011b). Interest in the beneficial rhizobacteria associated with cereals has increased recently and several studies clearly demonstrated the positive and beneficial effects of PGPR on growth and yield of different crops especially wheat at different environments under variable ecological conditions (Marques *et al*., 2010; Mehnaz *et al*., 2010; Zhang *et al*., 2012). Therefore, *Streptomyces* grow in a wide range of temperature which is aids the survival in the soil after application (Locci, 1994). A previous study conducted reported that Actinomycetes of *Streptomyces lydicus* species WYEC108 colonizes the roots of peas *Pisum sativum*. Such root colonizations are potentially important for the improvement and growth of *P*. *sativum* plant by improving the process of the nodule formulation of Leguminosae plants because it promotes nodule formation. Actinomycetes *Streptomyces lydicus* WYEC108 infected root by affecting root nodulation of pea *P*. *sativum* by increasing the frequency of root nodulation; it is thought to occur at the rate of Rhizobium sp. infection (Sahur *et al*., 2018). Soybean growth and production can be promoted by improving soil fertility in terms of physical-chemical and biological characterization. In order to achieve such condition, endophytic microorganisms such as Rhizobium and Actinomycetes should be utilized to promote the growth in the production of soybean plants (Sahur *et al*., 2018). Similarly, Dapiramicin produce by *Micromonospora* sp. is effective against Rice root disease (Ranjani, 2016).

### 2.7. Plant-microbe-interaction

Plant physiology is influenced by growth medium i.e., soil and in turn, plant roots modify the chemical, physical and biological properties of the soil. Plant roots deposit low molecular weight compounds such as amino acids, organic acods, sugars and other secondary metabolites in addition to high molecular weight compounds such as mucilage (polysaccharides) and proteins. It has been suggested that between 10% and 40% (or higher) of all fixed carbon can be secreted by the plant roots (Somers *et al*., 2004; Bais *et al*., 2006; Jones *et al*., 2009). The plant root exudates

shape a unique habitat, the rhizosphere, and select for microorganisms with key physiological capabilities (Ofek-Lalzar *et al*., 2014), thus leading to reduced microbial diversity (Minz *et al*., 2013; Ofek *et al*., 2014; Edwards *et al*., 2015). The rhizosphere is a highly dynamic and competitive microbial environment, and the composition of the root-associated microbial communities has been shown to be influenced mostly strongly by plant-derived carbon (Fatmawati *et al*., 2018). However, with greater distance from the roots, bulk soil microbial communities and organic carbon play a larger role in determining community composition (Inbar *et al*., 2005). It is a dynamic and complex micro environment inhabited by interactions of variety of microbes and plants. Rhizosphere is the major hub of the plant microbe interaction which is the determinants of plant health and soil fertility. Biofertilizers are living microbes which when added to plant supply nutrients e.g. root nodulating rhizobacteria, mycorrhizal fungi, several rhizobacteria like *Bacillus* spp. and rhizofungi like *Trichoderma* spp. They are able to solubilize plant-available phosphate and nitrogen from organic and inorganic bound phosphate and emit volatile organic compounds (Bitas, 2013). The microbial community of the root and surrounding soil has been shown to be shaped mostly by the soil type (Lundberg *et al*., 2012), plant host (Peiffer *et al*., 2013; Ofek *et al*., 2014; Ofek-Lalzar *et al*., 2014; Bulgarelli *et al*., 2015) and plant development stage (Lundberg *et al*., 2012). The root-associated microbial community is crucial for plant growth, supporting plant nutrition (Hamilton and Frank, 2001); (Bulgarelli *et al*., 2013), health (Santhanam *et al*., 2015) and stress tolerance (Lau and Lennon, 2012). It has also been demonstrated that plant-associated microbes can modify plant physiology, design root morphology (Zamioudis *et al*., 2013) and alter plant developmental processes, including flowering time and biomass accumulation (Panke-Buisse *et al*., 2015).

### 2.8. Physico-chemical soil characteristics

Agricultural crop productions and development of vegetation were based upon physico-chemical properties of the soil.The quality of the soil may analysedsoil parameters and processes which effects on soil to operate efficiently as a component of a sound ecosystem (Upadhyaya and Bajpai, 2010). Nitrogen is the most critical element obtained by plants from the soil and is a bottleneck in plant growth (Gorde,

2013). About 80% of the atmosphere is nitrogen gas. Nitrogen gas cannot taken by any plants directly where it can be “fixed” (converted) by nitrogen fixing microorganism. Phosphorus is important micro-nutrient present in every living cell (Tale and Ingole, 2015), essential for plant growth. Phosphorus most often limits nutrients remains present in plant nuclei and act as energy storage. Potassium is important element which plays an important role in different physiological processes of plants; it is one of the important elements for the development of the plant (Solanki and Chavda, 2012). This element involved in many plant metabolism reactions, ranging from lignin and cellulose used for the formation of cellular structural components, for regulation of photosynthesis and production of plant sugars that are used for various plant metabolic needs. The most significant property of soil is its pH level. It effects on all other parameters of soil. Therefore, pH is considered for analysis of any type of soil, where if the pH is less than 6 then it is said to be an acidic soil while the pH range from 6 to 8.5 it’s a normal soil and greater than 8.5 then it is said to be alkaline soil.

Due to fire, either as a normal or anthropogenic activity is likely to affect the microbial dynamics which in turn affects soil properties. Many species of actinomycetes that were isolated form the fired plots are being examined (Balser and Firestine, 2005; Hamman *et al*., 2007; Romanya *et al*., 2001; Zhou *et al*., 2009; Pandey *et al*., 2011). Traditional ecological knowledge implement that provides a strong relation between the cultural diversity and the biological diversity, and many contain valuable understandings for developing modern approaches that are technically sound as well as acceptable to the locals. The modern approaches may help to bring about much needed change by making use of global knowledge which is relevant locally (Ramakrishnan, 2009). Shifting cultivation has its own ecological merits, fallow helps in the restorations, conservation and the improvement of soil properties, e.g., addition of potassium to the soil during the process of burning, increase in soil pH and soil microbial biomass, suppression in the outgrowth of particular pest(s) and pathogen (s), least disturbance of the top soil, development of a good crop canopy due to mixed cropping, etc. (Paul and Paul, 2009). In Northeast India, the biological diversity of ecosystems are used and conserved by traditional

communities through various informal institutions and using traditional ecological knowledge. The survival of the growth promoting microbial species, after the slash and burn event of the shifting cultivation, looks to be clearly advantageous in management of agricultural crops that are grown in the fields after the completion of fire operations (Pandey *et al*., 2011). Mizoram is one of the biodiversity hotspots in the Indo Himalayan hilly region (21° 58' to 23° 36' N latitude and 92° 15' to 93° 29' E longitude) and is surrounded by other states viz., Tripura, Assam, and Manipur in north frontier regions, Bangladesh in west, and Myanmar in east and south. Shifting cultivation is a major agriculture land use practice in Mizoram due to its abundant forest land, low cost method, suitability to tropical soils and climate, and small farmer-friendliness. The total geographical area of Mizoram is 2.10 million ha, of which net sown area constitutes only 4.92% (0.10 million ha). Currently, 0.04 million ha of land is under shifting cultivation and forest cover is 75.6% (1.59 million ha) of the total area (Singh *et al*., 2013; Ibrahim *et al*., 2016).Shifting cultivation is found to be finest solution for agriculture in the humid tropics as long as the human population density is low and fallow periods are lengthy to restore soil fertility. An intensified practice of shifting cultivation (human-modified landscapes) results into destruction of natural vegetation, biodiversity, soil erosion, nutrient loss, and production of greenhouse gases during burning (Kuotsuo *et al*., 2014), posing a challenge to conservation practitioners for biodiversity persistence (Melo *et al*., 2013). In the past, shifting cultivation was sustainable and resourceful when population level was low and *jhum* cycle was long (15-30 years) to renew the soil fertility and stability (Ramakrshnan, 1992; Bruun *et al*., 2009). At present, the *jhum* cycle is shorthened to 3-5 years (Tawnenga and Tripathi, 1996; 1997a) which pose the problem of land degradation and threat to ecology in relation to soil erosion loss, low soil fertility, atmospheric smoke pollution, and reduction in crop yield (Tawnenga and Tripathi, 1997b) and sustainable capability of the habitat was lost in this system because of increasing intensity of land use (Grogan *et al*., 2012).

Plant growth promoting microorganisms play a major role in a very sensitive to ecological disturbances. PGP microbes have proved to be beneficial to soil health as well as increase the crop quality. Therefore, alternative biotechnological tactics are

reformed in different agriculture practices to not only increase the crop production and plant growth, but also tomaintain soil health (Fernando *et al*., 2005).

## 2.9. Significance of study

Understanding the diversity and distribution of indigenous actinobacteria in the rhizosphere of particular crops is depended on the knowledge of native actinobacterial populations, their isolation, identification, and characterization. It is therefore mandatory to explore region specific actinobacterial strains that can be used as growth promoters to achieve desired crop production (Deepa *et al*., 2010). However, a well-defined biodiversity and taxonomic study of actinomycetes is important to understand actinomycetes from the unexplored environment (Jensen, 2010). It is well known that microbial diversity has not been efficiently explored and the vast majority of prokaryotes (90-99%) present in natural habitats are still to be isolated (Harwani, 2013). Many natural environments are still either unexplored or underexplored and thus can be considered a prolific resource for the isolation of poorly studied microorganisms including rare actinomycetes (Tiwari and Gupta, 2012). Many extremophilic bacteria are recognized to be of industrial interest as potential candidates for future biotechnological applications (Cayol *et al*., 2015). Actinomycetes were isolated from various important medicinal plants. (Gopinath *et al*., 2018) isolated antibiotic producing actinomycetes from the rhizosphere soil of *Cipadessa baccifera* and *Clausen adentata.* (Raut and Kulkarni, 2018) were isolated actinomycetes from the rhizosphere soil of medicinal plants viz; *Aloe barbadense*, *Emblica officinalis*, *Zingiber officinale*, *Tinospora cardifolia*, *Nerium oleander*, *Eucalyptus camaldulensis*, *Mentha arvensis*, *Santalum album*, *Hibiscus – rosa-sinensis*, *Ocimum sanctum* and *Curcuma longa*. (Darshit and Pandya, 2018) isolated from medicinal plants viz, *Aloe vera*, *Azadirachta indica*, *Syzygium cumini*, *Datura stramonium*, *Rosa indica*, *Pongamia pinnata*, *Oscimum sanctum*, *Allium sativum*, *Allium cepa*, *Trigonellafoenum-graecum* and *Psoralea corylifolia*.

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# Chapter 3

## Materials and methods

### 3.1. Study sites and the collection of samples

In order to explore the actinomycetes associated with the major crop plants under shifting cultivation of Mizoram, Northeast India. Rhizospheric samples were collected from two *jhum* cultivation areas of Mizoram i.e., Reiek (at 23˚68'04.138''N and 092˚62'70.182''E, elevation of 1465) and Tanhril (at 23044´55.25´´ N and 092038´36.68´´ E, elevation has 535 m) (Fig.1). Reiek is a mountain with maximum peak at an m situated about 29 km from Aizawl surrounded by various plant species, valleys and hills. Tanhril is situated in the central part of the Aizawl district and located at 15 km from Aizawl, Mizoram. Soil samples were collected from the roots of major crops viz., rice, maize, brinjal, yam, chilly and bean under jhum cultivation in Reiek and Tanhril, Mizoram. Soil samples were taken from each plant by uprooting the whole crop plant and gentle shaking on the plane paper, and any strongly adhered particles were removed with the help of tweezers without disturbing the plant. Collected rhizospheric soil samples from different crop plants of each study area were then mixed together in sterile universal containers and labeled separately for both study areas. After collection samples were brought to the Laboratory. Collected soils were stored in the laboratory at 4˚C for further use (Kasa *et al*., 2015).

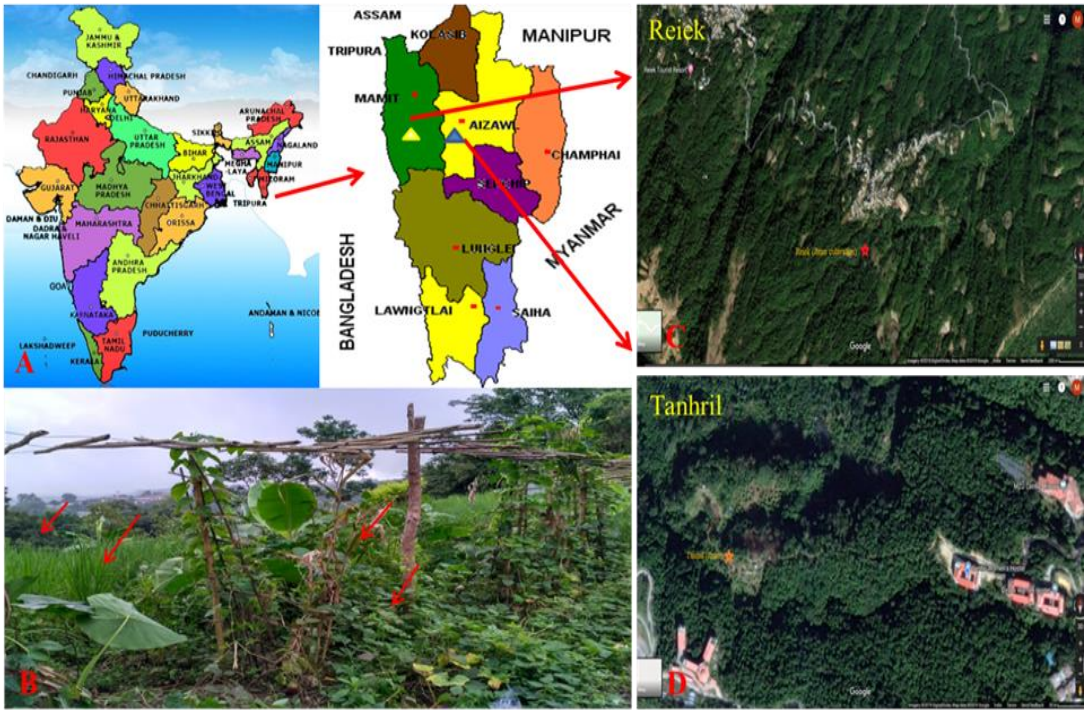


**Fig.3.1:** A. Map of study area B. Major crops of shifting cultivation C. Reiek shifting cultivation D. Tanhril shifting cultivation

### 3.2. Isolation of rhizosphere actinomycetes from soil samples

1g of each soil samples were dried in a laminar flow-hood overnight. Dried samples were crushed into a fine powder using sterile mortar and pestle and sieved to exclude large minerals and organic particles. The soil samples were further treated with selective pre-treatment of soil prior to isolation of actinomycetes; i.e. (i) heating at 70˚C of temperature for 20 mins (Seong *et al*., 2001) followed by (ii) rehydration in the moist incubation and centrifugation (Hayakawa *et al*., 1997). Pre-treated soil was then dissolved in 0.9% saline water (NaCl) and serially diluted down to 10-6 concentration.

Then, 100 µl aliquots of the diluted samples from each dilution was plated on prepared medium, supplemented with Nalidixic acid (50µg/ml) dissolved in Chloroform and Amphothericin B (50µg/ml) dissolved in Dimethyl sulfoxide (DMSO). Four isolation media were used to isolate soil actinomycetes. The basal composition of these media were: (a) International Streptomyces Project 2 [ISP2] (Yesat extract 4g, Malt extract 10g, Dextrose 4g, Agar 20g, pH 7.2), (b) Starch

Casein Agar [SCA] (Starch 10g, Casein 1g, $KNO_3$ 2g, $KH_2PO_4$ 2g, NaCl 2g, $MgSO_4.7H_2O$ 0.5g, $CaCO_3$ 0.02g, $FeSO_4.7H_2O$ 0.001g, Agar 18g, pH7.0-7.4), (c) Cross Streak Media [CSM] (Yesat extract 3g, Peptone 3g, Casein 3g, Starch 8g, $K_2HPO_4$ 0.5g, $MgSO_4.7H_2O$ 0.5g, NaCl 2g, Agar 15g, pH 7.0-7.6) and d) IM8 media (Glucose 10g, peptone 5g, Tryptone 3g, Nacl 5g, Agar 15g, pH 7.0). All the above-mentioned compositions were for one litre of media. Inoculated plates were incubated at 28 ± 2°C for 1-4 weeks. Representatives of isolates putatively assigned as actinomycetes were picked randomly from the plates using sterile toothpicks and streaked onto their respective media plates to obtain pure culture of the strains. The isolates were incubated at 28 ± 2°C for 10 to 14 days. Pure cultures of the isolates were finally maintained as 25% glycerol stock at -80°C.

### 3.3. Morphological and Microscopic characterization

Visual observation of both morphological and microscopic characteristics like aerial mycelia, spore distinctive reverse colony color, color of diffusible, spore chain morphology etc. were studied (Thampayak *et al*., 2008). The spore chain morphology and surface of spore were examined by electronic microscope of 10-day old cultures grown on International Streptomyces Project 1 (ISP1) media. The morphological identification of the isolates were followed the keys of Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 2000).

### 3.4. *In-vitro* screening of rhizospheric actinomycetes isolates for plant-growth promoting properties

#### 3.4.1. Screening for phosphate solubilization

Qualitative phosphate solubilization activity of rhizospheric actinomycetes isolates was carried out following standard methods (Doumbou *et al*., 2001). Rhizospheric actinomycetes isolates were inoculated on Pikovskaya's medium and incubated at 28°C for seven days. The halo zone around the colony was presumptive confirmation of phosphate solubilization.

#### 3.4.2. Indole-3-acetic acid (IAA) production

The production of IAA by rhizospheric actinomycetes isolates was estimated according to Gordon and Weber (1951). The isolates were grown on ISP1 broth

containing 0.2% filter sterilized (0.22µm membrane filter) L-tryptophan solution and incubated at 28°C with continuous shaking at 125 rpm for seven days at 28°C. Cultures were centrifuged at 11,000 rpm for 5min. 1mlof the supernatant was mixed with 2 ml of the Salkowski reagent (1 ml of 0.5M $FeCl_3$ in50 ml of 35% $HClO_4$) and incubated in dark for half an hour. Development of pink colour indicated the production of IAA.

### 3.4.3. Siderophore production

Siderophore production of the rhizosphere actinomycetes isolates was determined by the method of Schwyn and Neilands (1987). A loop full of culture was inoculated on Chrome azurol S (CAS) agar medium and incubated at 28± 2°C for 5 d. The colony with a halo zone of yellow-orange color was considered positive for siderophore production.

### 3.4.4. Ammonia production

The rhizospheric actinomycetes were tested for ammonia production described by Cappucino and Sherman (1992). Culture was inoculated in peptone water and incubated at 30±2°C with shaking at 120 rpm for 3 weeks. 0.5 ml of Nessler's reagent was added into 10 ml of the culture. Development of brown to yellow color was recorded as a positive test for ammonia production.

### 3.4.5. Nitrogen fixation

Isolates were inoculated on Nitrogen free medium plates with 0.05% (w/v) bromothymol blue indicator and observed for change in colour from yellowish to green (Nakbanpote *et al*., 2013).

### 3.4.6. Amylase production

Screening of amylase producers done by following the method of Kasana *et al*. (2008).Few colonies of test organism were picked and were streaked onto a starch plate in the form of a line across the width of the plate. Several cultures were tested on a single agar plate in similar manner, each represented by a line at 28 °C for 7 days. Addition of 2-3 drops of iodine solution onto the edge of colonies will produce clear halo zones around the colonies within 10-15mins indicating positive result.

### 3.4.7. Catalase production

Culture was grown in a test tube at 28°C for 7 days. A drop of 3% hydrogen peroxide (H2O2) wasadded to 5ml culture at once and observed for effervescence (Singh and Padmavathy, 2014).

## 3.5. *In-vivo* screening of actinomycetes for PGP potential

### 3.5.1. Preparation of culture (AB832)

The most PGP potent actinomycete isolate was selected for pot experiment. Isolate was grown in its respective media broth at 28˚C for 7 days with continuous shaking at 120rpm. The O.D. of the suspension culture was measured at 600nm using a spectrophotometer during this time interval. When it reached 0.6 O.D., it was taken out and stored at 4˚C for latter pot experiments.

### 3.5.2. Pot experiment

Soils for pot experiment were collected from the *jhum* cultivation area. Collected soil was divided into two parts. One part was sterilized (treatment) and other part remained as unsterilized soil (control). The selected actinomycetes strain AB832 was evaluated for PGP potential on *Zea mays* L. (locally called Mimpui), and *Phaseolus mungo* L. (Zorin) collected from the local cultivators. The maize and bean seeds were sown under sterilized non-sterilized soil (250g each pot) with pot size of 7 x 7.62 cm and treated with AB832. Eight seeds were sown in each pot. The treatment pots treated with suspension of isolate AB832 ($10^{-6}$ CFUmL-1) and the control pots contained no AB832 culture. Plants were grown in the mist chamber with twelve replicates for each plant, inoculated with organism once in 3 days, and watered daily with normal water until plants were harvested. Germination percentage was recorded after 5 days while after 15 days, plants from each treatment were uprooted and measurements were taken for length of shoot and root, fresh weight and dry weight of the whole plant. Seed germination percentage and estimation of shoot and root were studied by software winRhizo2012b. Data were statistically analyzed by Microsoft excel using a one-way ANOVA and LSD tests at $p < 0.05$.

### 3.5.3. Rhizospheric soil characteristics

The treatments of rhizospheric soil from bean and maize were used to evaluate their chemical properties after 15 days. The treated plants were uprooted gently and rhizospheric soils were collected from each plant without damaging the root system under greenhouse condition. Briefly, eight plants were grown in each pot filled with the 3 years old *jhum* cultivation soil. The rhizosphere soil samples were collected from soils adhering to plant roots. Total of 10 composite samples were collected and brought to the laboratory, and passed through a 2-mm sieve, stored at 4°C. Soil pH, soil organic carbon (SOC), available nitrogen (N), available phosphorus (P), and available potassium (K) were analysed in the laboratory at KVK, Kolasib, Mizoram.

## 3.6. Genomic DNA extraction, amplification of 16S r RNA gene and sequencing

Total genomic DNA was extracted using Soil DNA extraction Kit (Invitrogen). The DNA purity was quantified by absorption spectrophotometry at 260 and 280nm and concentration were measured. 16S r RNA gene sequence was amplified by using forward primerAB1 (5′AGTGGCGAACGGGTG3′) (Sengupta *et al*., 2015) and reverse primer 1378R (5′CGGTGTACAAGGCCC GG3′) (Heuer *et al*., 1997). The PCR reaction was performed in a final volume of 25 µl, which consisted of template DNA 2 µl: molecular grade $H_2O$: 15 µl, buffer: 3.5 µl including 12.5 mMMg$Cl_2$, 1 µldNTP mix (10 mM each nucleotide), 1 µl of forward and reverse primer each (concentration 10 picomole), 0.5 µl (2U) of Taq-polymerase and 1 µl DMSO under the following cycling conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 60 s, annealing at 54 °C for 50 s,72 °C for 120 s and final extension of 72 °C for 10 min. PCR products were purified with QIA quick PCR cleanup kit (Qiagen). PCR-amplified templates (10µl) were sequenced using ABI 3100 Genetic Analyzer (Applied Biosystems). The sequences generated were compared with Gen Bank database using Blast N for searching the closest match sequence. The sequences were pairwise aligned using the program Clustal W packaged in the MEGA 7.0. software (Thompson *et al*., 1997).

### 3.7. Phylogenetic analysis

The Sequences generated after sequencing of the 16S rRNA gene was compared with GenBank database using BlastN for searching the closest match sequence. The sequences were pairwise aligned using the program Clustal W packaged in the MEGA 7. software (Kumar *et al*., 2016). The data obtained was used to derive phylogenetic tree with the same softwere and a Neighbour Joining tree was generated (Saitou and Nei, 1987). Bootstrap analyses with 5,000 resamplings was performed with MEGA 7 using p-distance model (Felsenstein, 1985).

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# Chapter 4

# Results

## 4.1. Isolation of rhizopsheric actinomycetes

The rhizospheric actinomycetes from the major crop plants of two different shifting cultivation (*jhum*) areas (Reiek and Tanhril) were isolated and analysed. Isolated rhizospheric actinomycetes were screened *in-vitro* for their PGP properties like phosphate solubilization, nitrogen fixation, ammonia, and IAA, amylase and catalase productions. The isolates *in-vitro* positive PGP characterization were subjected to a comprehensive in-*vivo* screening for various plant-growth promoting (PGPRs) traits and their responses to soil chemical properties of plant rhizosphere on bean and maize crop plants of Mizoram with reference to *jhum* cultivation soil under control condition. Further PGP potential rhizopsheric actinomycetes were analysed for their 16S rRNA sequencing and phylogeny.

A total of 35 rhizospheric actinomycetes were obtained in the present study. Out of total, 32 (91.4%) actinomycetes were isolated from *jhum* cultivation of Reiek and the remaining 3 from *jhum* cultivation of Tanhril (Fig. 4.1). Based on media employed, 12 isolates (34.2%) were able to grow in CSM followed by 11 isolates (31.4%) in SCA media, 8 isolates (22.8%) were obtained from ISP2 media and 4 isolates (11.4%) from IM8 media (Fig. 4.2).

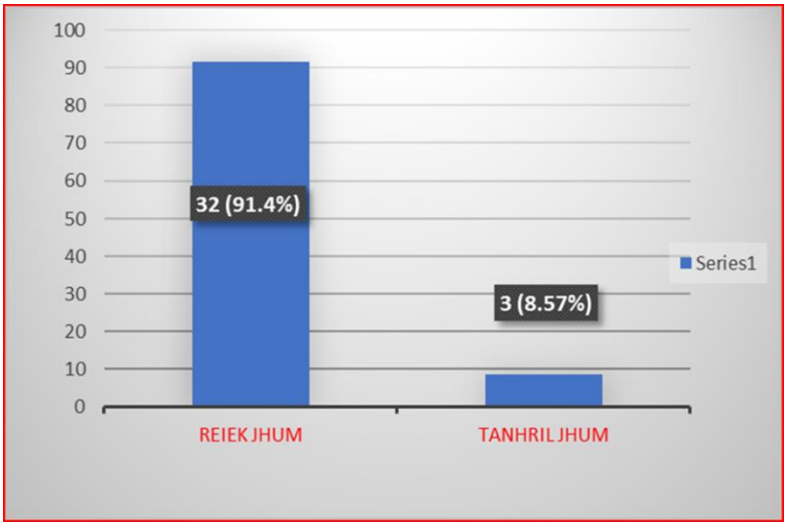


**Fig.4.1:** Distribution of total isolates from two study areas

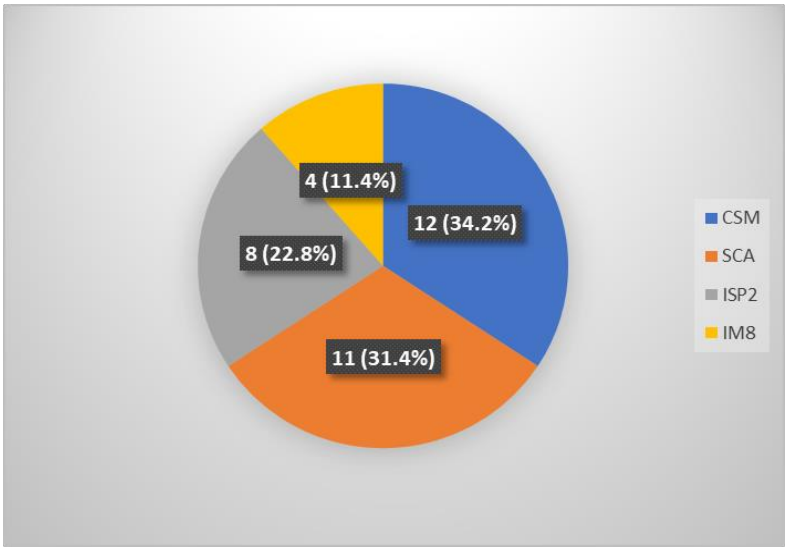


**Fig.4.2:** Distribution of isolates in various media employed

## 4.2. Characterization and identification of rhizospheric actinomycetal isolates

Microorganisms actinomycetes can be classified based on their colony characteristics, pigment production and growth formation. Bergeys Manual of Determinative Bacteriology is routinely used for classification of the bacteria and actinomycetes. The colonies of rhizospheric actinomycetes isolates were examined morphologically for their shape, size, margin, elevation, appearance, texture, pigmentation, and optical properties (Fig. 4.3). In addition, cellular morphology, shape, gram staining was also examined under the microscopy of 100X

magnification (Fig. 4.4). The images of the actinomycetal isolates showed the presence and absence of aerial and substrate mycelium, fragmentation of the substrate mycelium, presence of sclerotia or sporangia and sporulation pattern and spore chain morphology. Based on colony and cultural characteristics, total 35 actinomycetes were identified. The most frequently isolated actinomycetes species was *Streptomyces* followed by *Micromonospora* sp. from the crop plants (Table 4.1., fig 4.3.). In this study, rhizopsheric actinomycetes were isolated from collected mixer of soil of major crop plants viz., rice, maize, bean, yam, chilly and brinjal cultivated under *jhum* cultivation. The isolates were cultured on the selective medium for actinomycetes i.e., Starch Casein Agar (SCA), Cross-Streak Media (CSM), International Streptomyces Project 2 (ISP2) and IM8 media. Inoculated culture plates were incubated in BOD at 28˚C±2˚C for 3 to 31 days. Actinomycetes grow within 3 - 7 days were considered as fast-growth and those grow within 7-31 days were considered as slow-growth actinomycetes. When full-grown on an agar-surface, the actinomycetes branch making a network of hyphae growing both on the surface and under-surface of the agar were observed. The colour of the aerial mycelium of actinomycetes were observed as: off-white, yellow, deep orange, light cream, cream, grey, white and very light colour. Substrate mycelium was mostly cream, orange, yellow and brown (Fig. 4.3). Yellow pigmentation for strains AB728, AB734 and AB778 were recorded (Fig. 4.3). Maximum isolates were sticky, very sticky, sticky-hard, and hard in nature and colony with 0.3mm to 1.5mm in sizes. Actinomycetes colonies were entire, irregular, filamentous, circular and convex, raised, flat, umbonate, undulate forms. On culture media, rhizospheric actinomycetes production of earthy or smutty odour was noted.

**Table 4.1:** Colony morphological characteristics of the total rhizosphere actinomycetal isolates

| Sl. no. | Isolate code | Source of isolation | Morphology | Media used | Identified organism |
|---|---|---|---|---|---|
| 1. | AB726 | Tanhril *jhum* | Deep orange (AM), orange (SM); sticky-hard; colony are entire and raised; tiny in size; growth in 31 days | CSM | *Micromonospora auratinigra* |
| 2. | AB727 | Tanhril *jhum* | Light cream, entire, raised and very sticky in nature (AM); yellow in colour (SM); growth in 10 days | IM8 | *Streptomyces* sp. |
| 3. | AB728 | Tanhril *jhum* | Off-white, entire, convex and very sticky (AM); yellow (SM); yellow pigment; 0.6mm in dia., growth in 10 days | ISP2 | *Streptomyces* sp. |
| 4. | AB729 | Reiek *jhum* | Off-white and grey, umbonate form and very hard (AM); green-yellow (SM); 0.5mm in dia., growth in 7 days | ISP2 | *Streptomyces* sp. |
| 5. | AB732 | Reiek *jhum* | Off-white and light orange, raised and jelly-like nature (AM); yellow and orange (SM); 0.5mm in dia., growth in 7 days | SCA | *Streptomyces* sp. |
| 6. | AB733 | Reiek *jhum* | Pure grey, entire, raised, smooth and sticky nature (AM); dark brown (SM); 0.7mm in dia., growth in 7 days | SCA | *Streptomyces* sp. |
| 7. | AB734 | Reiek *jhum* | Pure grey and white-powdery on top, entire and flat (AM); yellow (SM); yellow pigment; growth in 7 days | CSM | *Streptomyces* sp. |
| 8. | AB737 | Reiek *jhum* | Grey, flat and sticky form (AM); brown (SM); growth in 10 days | CSM | *Streptomyces* sp. |
| 9. | AB738 | Reiek *jhum* | Light colour, compact and jelly form (AM); yellow and orange (SM); growth in 5 days | CSM | *Streptomyces* sp. |
| 10. | AB742 | Reiek | Light grey, irregular, undulate and | CSM | *Streptomyces* sp. |

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|---|---|---|---|---|---|
| | | *jhum* | sticky nature (AM); 1mm in dia., growth in 7 days | | |
| 11. | AB744 | Reiek *jhum* | Off-white, circular (AM), cream (SM), sticky, colony with 2mm in dia. | CSM | *Streptomyces* sp. |
| 12. | AB746 | Reiek *jhum* | Off-white, circular, lobate (AM); yellow and orange (SM); colony with 1.5mm in dia., growth in 10 days | CSM | *Streptomyces* sp. |
| 13 | AB757 | Reiek *jhum* | Light colour (AM), dark cream (SM); sticky-rough; colony are irregular and convex; colony with 0.4mm in dia.; growth in 7 days | CSM | *Streptomyces venezuelae* |
| 14. | AB759 | Reiek *jhum* | Light grey-white (AM), dark yellow-brown (SM); soft; powdery and filamentous; growth in 5 days | CSM | *Streptomyces venezuelae* |
| 15. | AB761 | Reiek *jhum* | Light colour (AM), cream (SM); tiny in size; very sticky; raised; growth in 7 days | CSM | *Streptomyces avellaneus* |
| 16. | AB763 | Reiek *jhum* | Light grey and off-white, entire, umbonate and sticky-hard (AM); cream (SM); 1mm in dia., growth in 10 days | CSM | *Streptomyces* sp. |
| 17. | AB770 | Reiek *jhum* | Off-white (AM), cream-orange (SM); hard; entire and convex; colony with 1.1mm in dia.; growth in 7 days | CSM | *Streptomyces seoulensis* |
| 18. | AB773 | Reiek *jhum* | Off-white, very-hard, irregular and curled (AM); yellow and orange (SM); 0.5mm in dia., growth in 10 days | ISP2 | *Streptomyces* sp. |
| 19. | AB774 | Reiek *jhum* | Off-white (AM), yellow (SM); hard; entire and umbonate; colony with 1.2mm in dia.; growth in 7 days | ISP2 | *Streptomyces scabiei* |
| 20. | AB777 | Reiek *jhum* | Off-white, irregular, filamentous and sticky (AM); bright yellow | SCA | *Streptomyces* sp. |

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|---|---|---|---|---|---|
| | | | (SM); 1mm in dia., growth in 5 days | | |
| 21. | AB778 | Reiek *jhum* | Off-white, entire, convex, very-hard (AM); yellow (SM); yellow pigment; 0.4mm in dia., growth in 15 days | ISP2 | *Streptomyces* sp. |
| 22. | AB781 | Reiek *jhum* | Yellow, entire, convex and jelly (AM); dark yellow (SM); 0.8mm in dia., growth in 7 days | IM8 | *Streptomyces* sp. |
| 23. | AB782 | Reiek *jhum* | Deep off-white (AM), light yellow (SM); sticky-gel; irregular and raised; colony with 0.3mm in dia.; growth in 5 days | SCA | *Streptomyces vinaceus* |
| 24. | AB784 | Reiek *jhum* | Off-white (AM), yellow (SM); hard; irregular, wrinkle and umbonate; colony with 1.2mm in dia.; growth in 7 days | ISP2 | *Streptomyces scabiei* |
| 25. | AB786 | Reiek *jhum* | Off-white (AM), cream (SM), sticky-hard, punctiform, raised, colony with 0.5mm in dia. | SCA | *Streptomyces*sp. |
| 26. | AB788 | Reiek *jhum* | Off-white and on top grey (AM), dark yellow and green in the middle of the colony (SM); hard; circular and umbonate; colony with 1.2mm in dia.; growth in 7 days | ISP2 | *Streptomyces* sp. |
| 27. | AB795 | Reiek *jhum* | Grey and off-white, irregular, umbonate, very hard (AM); dark yellow (SM); 1mm in dia., growth in 10 days | ISP2 | *Streptomyces* sp. |
| 28. | AB796 | Reiek *jhum* | Off-white, powdery, flat (AM); yellow (SM); growth in 5 days | SCA | *Streptomyces* sp. |
| 29. | AB797 | Reiek *jhum* | White, filamentous and sticky-hard (AM); cream-orange (SM); 1.5mm in dia., growth in 15 days | SCA | *Streptomyces* sp. |
| 30. | AB805 | Reiek *jhum* | Off-white, irregular, crateriform and sticky-hard (AM); dark yellow (SM); yellow pigment; 1.5mm in | SCA | *Streptomyces* sp. |

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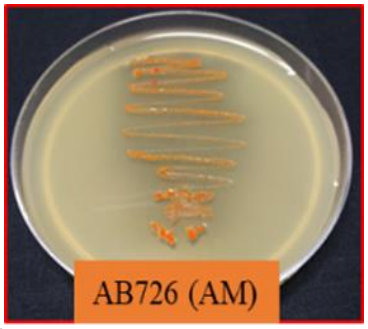
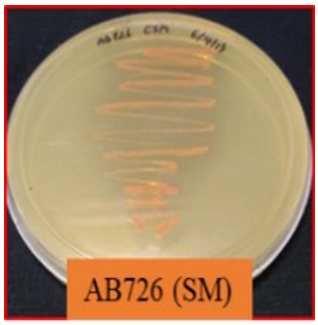
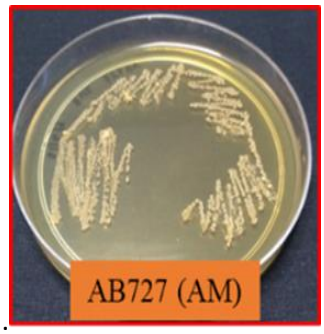
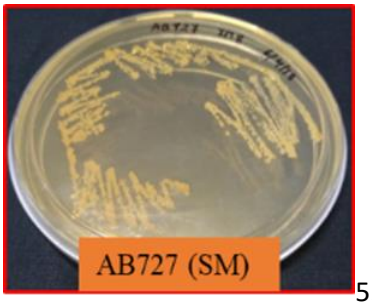
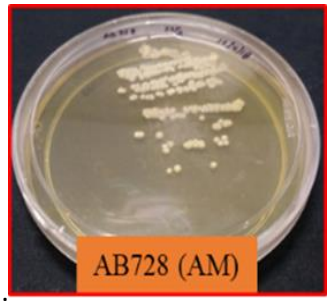
|---|---|---|---|---|---|
| | | | dia., growth in 7 days | | |
| 31. | AB812 | Reiek *jhum* | Off-white, colony with ring, entire, umbonate and very hard (AM); cream (SM); 0.4mm in dia., growth in 15 days | SCA | *Streptomyces* sp. |
| 32. | AB816 | Reiek *jhum* | Off-white, irregular, undulate and sticky-hard (AM); dark cream-orange (SM); 1mm in dia., growth in 5 days | SCA | *Streptomyces* sp. |
| 33. | AB822 | Reiek *jhum* | Light in colour, irregular and jelly (AM); cream (SM); growth in 5 days | CSM | *Streptomyces* sp. |
| 34. | AB828 | Reiek *jhum* | Off-white and ring (AM), cream (SM); hard in nature; circular and umbonate; colony with 1mm in dia.; growth in 7 days | SCA | *Streptomyces mirabilis* strain |
| 35. | AB832 | Reiek *jhum* | Irregular, raised and undulate form of colony; growth in 7 days | SCA | *Streptomyces* sp. |

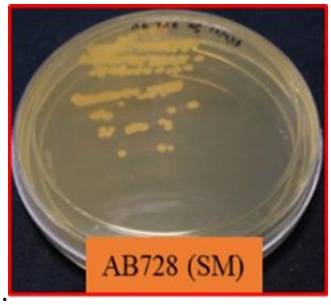
1. 

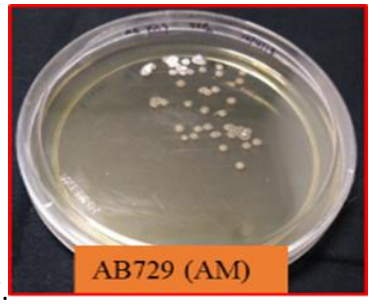

2. 

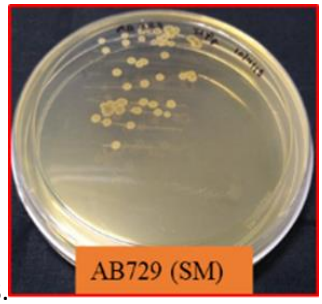

3. 

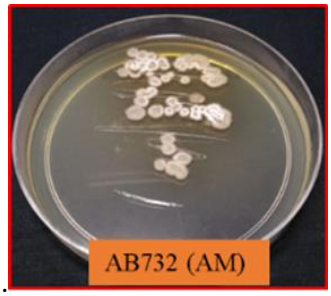

4. 

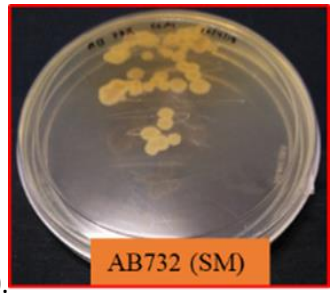

5. 


6. 


7. 


8. 


9. 


10. 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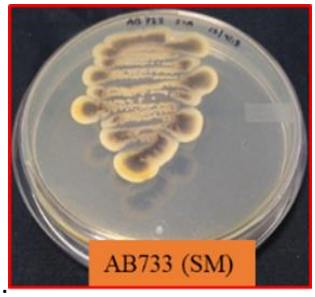
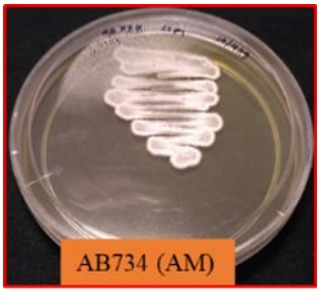
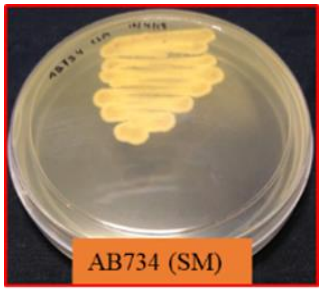
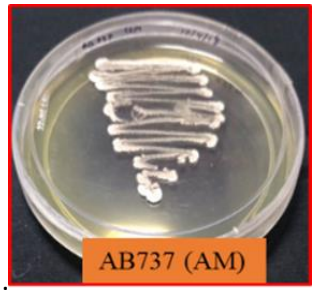
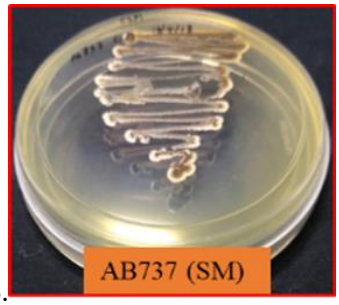
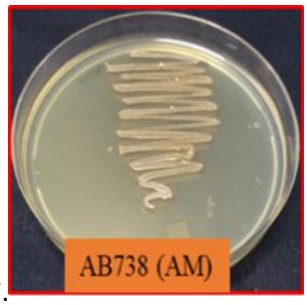
12. 

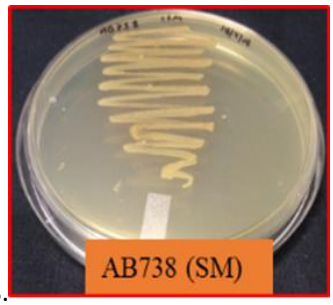

13. 

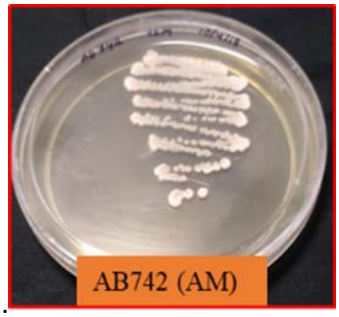

14. 

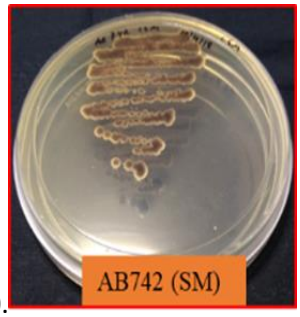

15. 

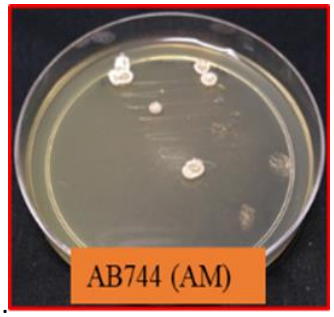
16. 

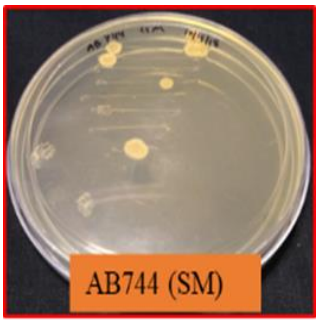
17. 

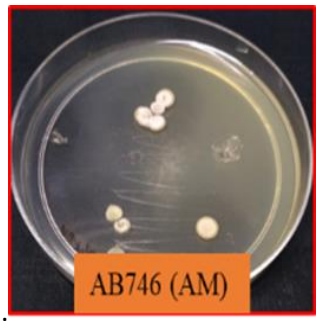
18. 

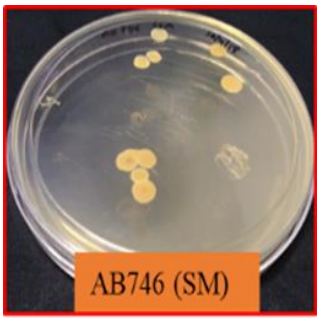
19. 

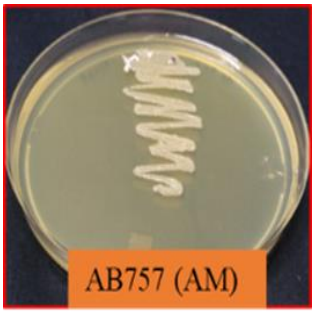
20. 

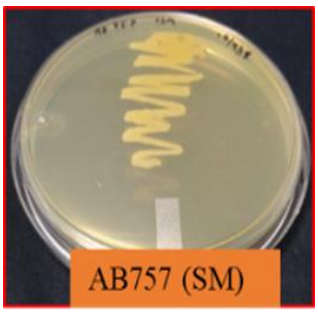
21. 

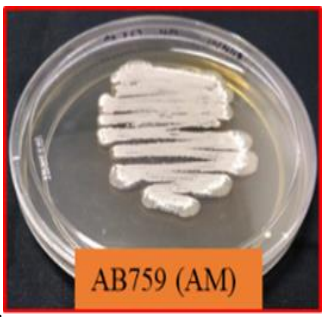
22. 

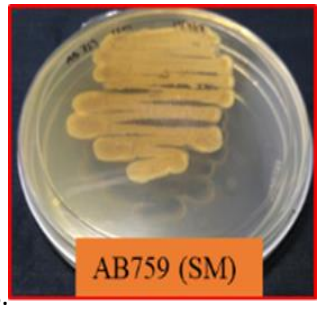
23. 

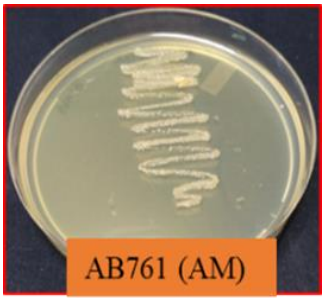
24. 

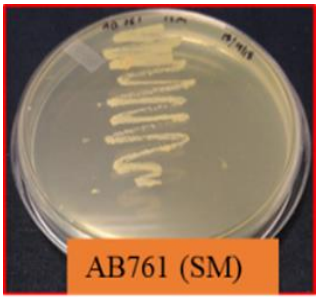
25. 

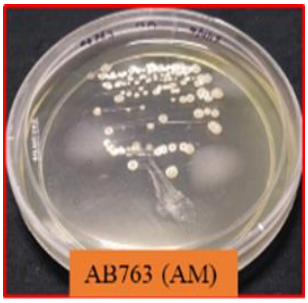
26. 

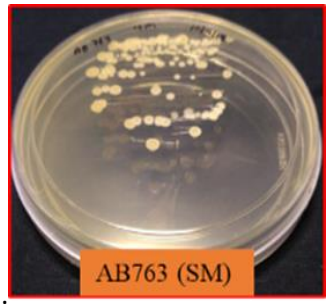
27. 

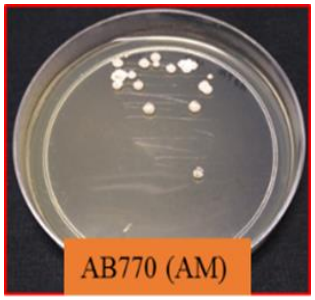
28. 

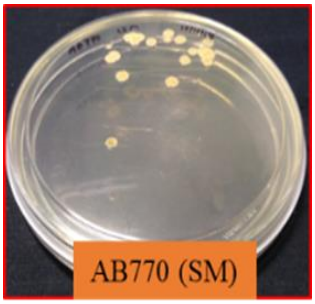

29. 

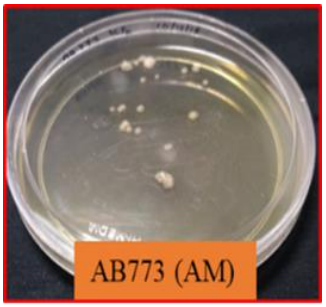

30. 

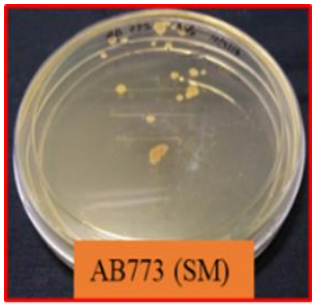

31. 

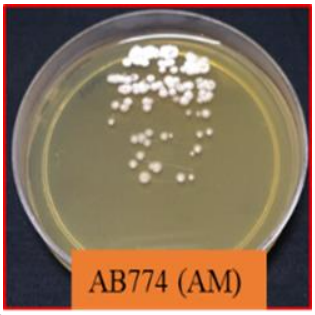

32. 

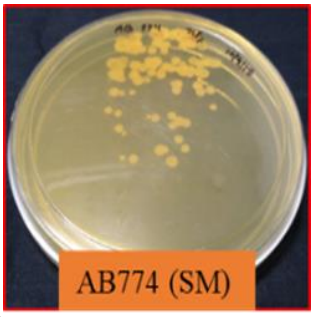

33. 


34. 


35. 


36. 


37. 


38. 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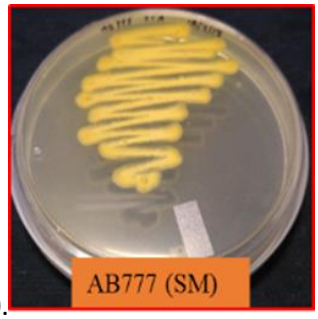
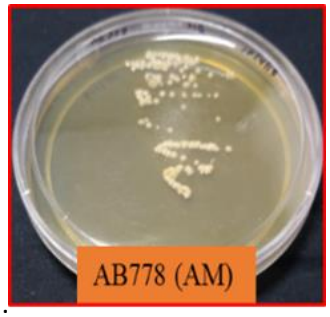
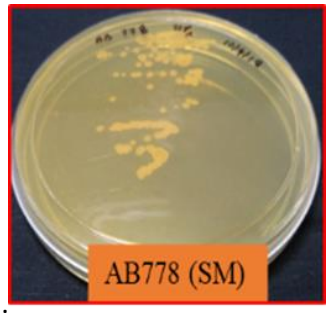
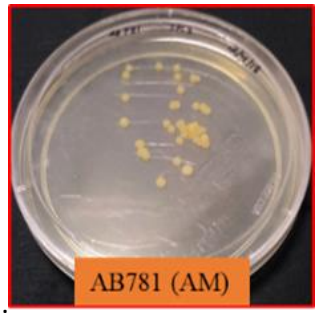
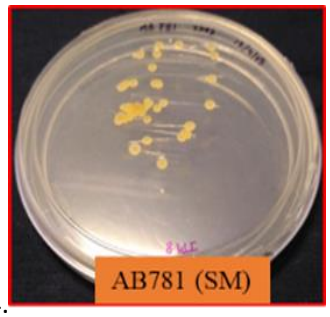
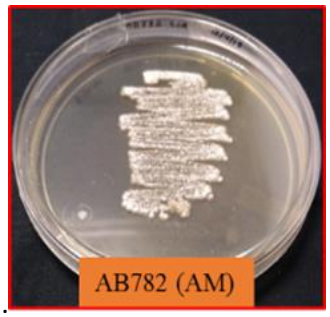
40. 

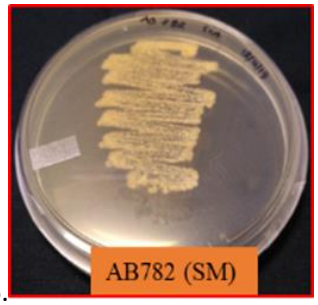

41. 

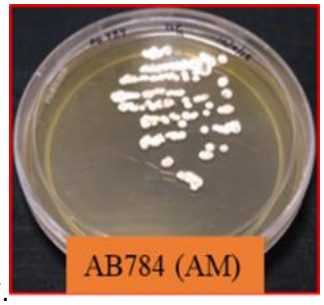

42. 

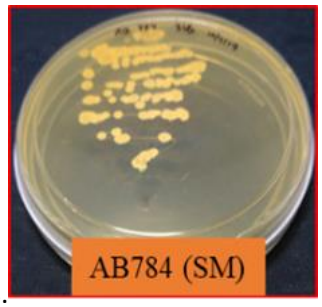
43. 

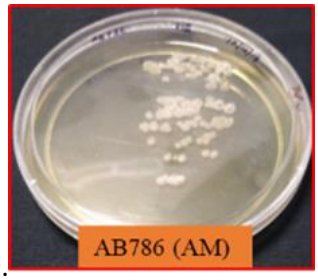
44. 

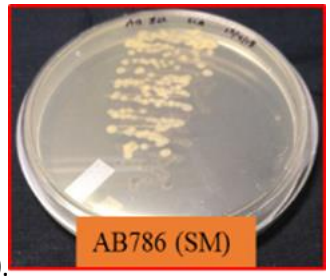
45. 

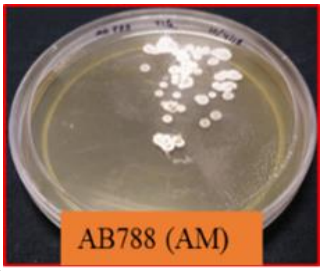
46. 

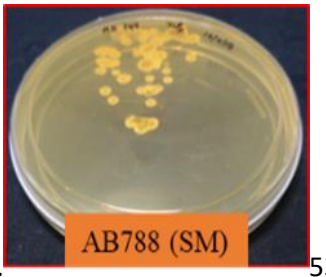
47. 

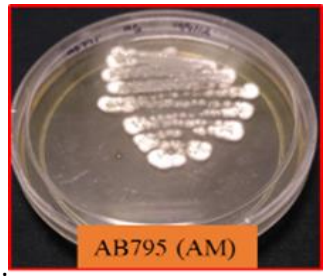
48. 

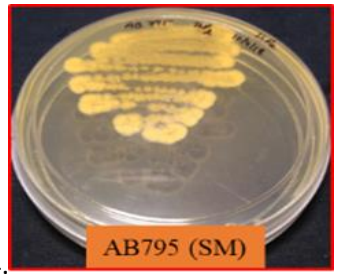
49. 

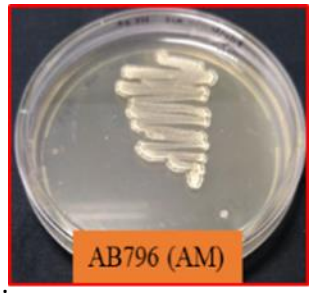
50. 

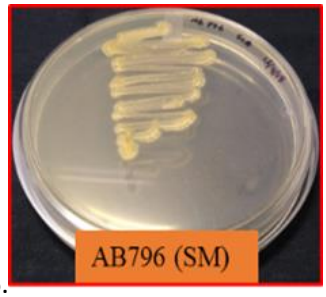
51. 

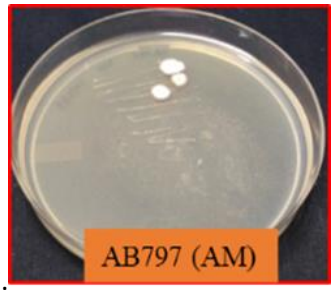
52. 

53. 

54. 

55. 

56. 

57. 

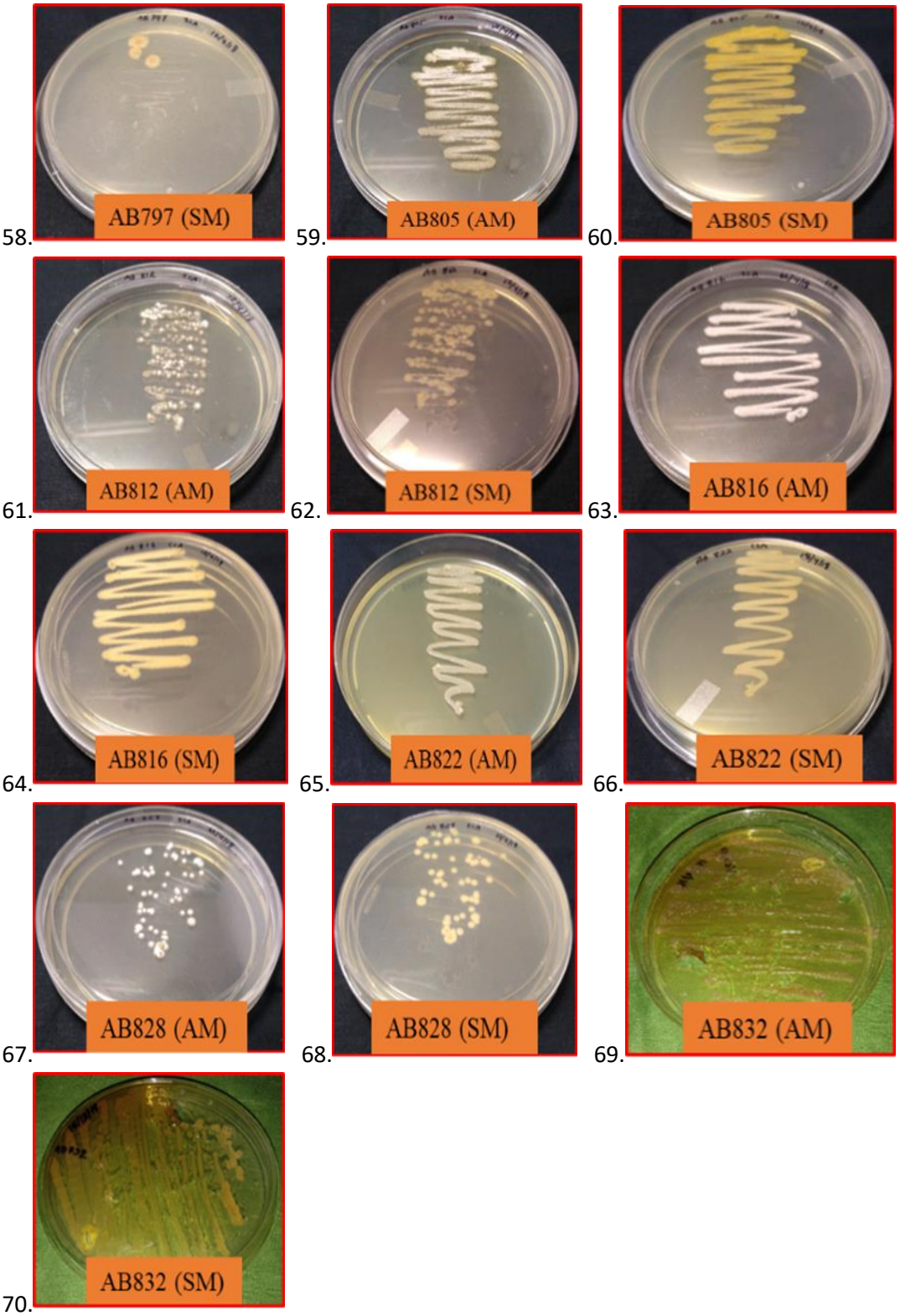



**Fig.4.3:** Total 35 rhizospheric actinomycetes isolated from major crops of *jhum* cultivation (AM were indicative of Aerial Mycelium; SM were indicative of Substrate Mycelium)

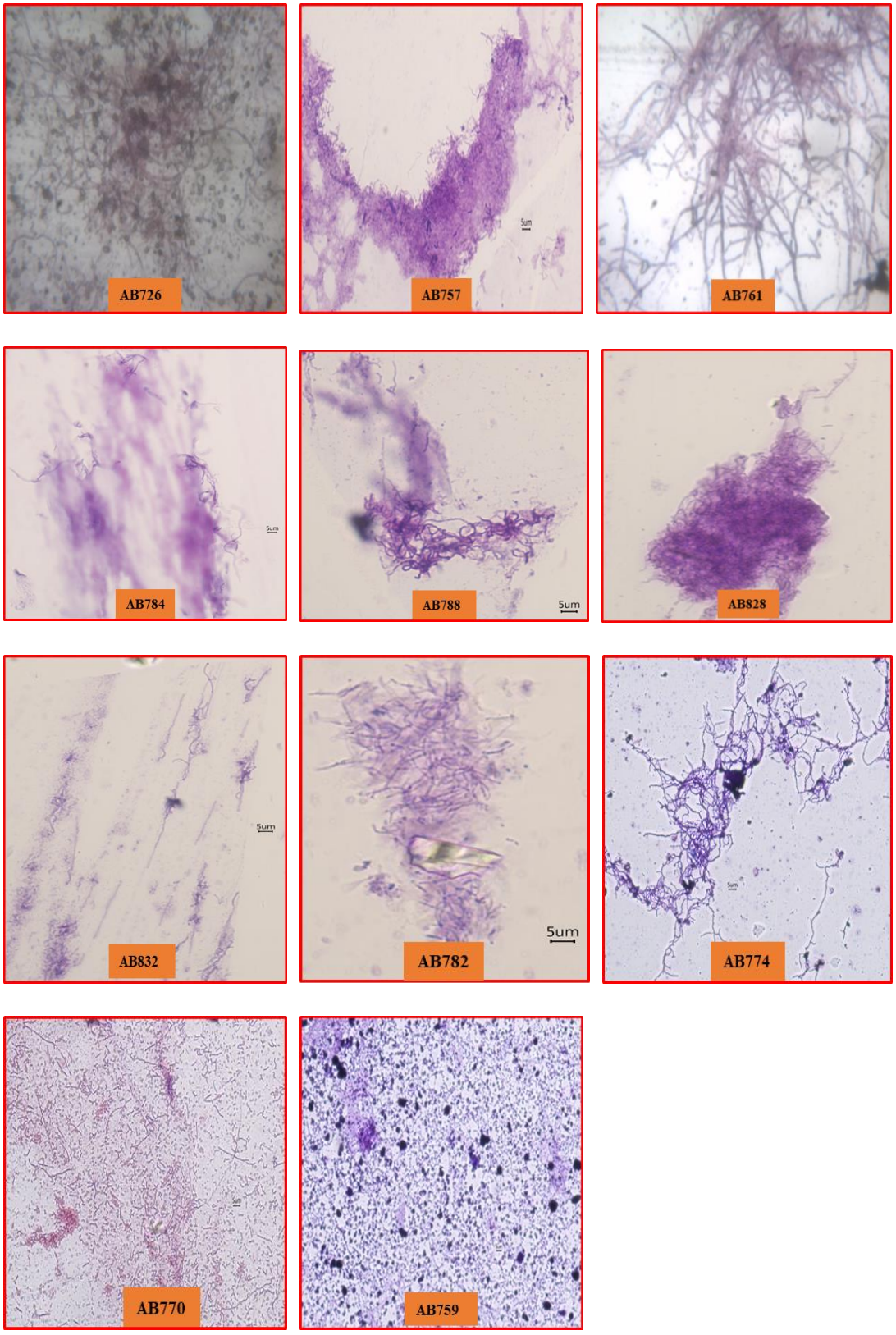



**Fig.4.4:** Microscopical morphology of total 11 potential PGP rhizospheric actinomycetes

### 4.3. *In-vitro* screening of the isolated rhizospheric actinomycetes for plant-growth promoting potential

Morphologically identified rhizospheric actinomycetes were tested *in-vitro* for their abilities to promote plant growth. Among 35 isolates, 11 strains were able to show PGP properties.

#### 4.3.1. Phosphate solubilization

The ability of selected strains to solubilize inorganic phosphate from the media was tested. Among the 35 strains, 11 (AB726, AB757, AB759, AB761, AB770, AB774, AB782, AB784, AB788, AB828, AB832) isolates were able to solubilize phosphate and formed clear zones on modified Pikovskaya agar plates (Table 4.2).

#### 4.3.2. Ammonia production

The rhizospheric actinomycetes isolates was tested for the production of ammonia. Culture was inoculated in peptone water and incubated at 30±2◦C with shaking at 120 rpm for 3 weeks. A 0.5 ml of Nessler's reagent was added into 10 ml of the culture. Development of brown to yellow colour was recorded as a positive test for ammonia production. Out of 35, 2 strains (AB759 and AB832) isolate showed ability to produce ammonia. Maximum ammonia production was recorded in Strain AB832 and minimum in strain AB759 (Table 4.2., Fig. 4.5).

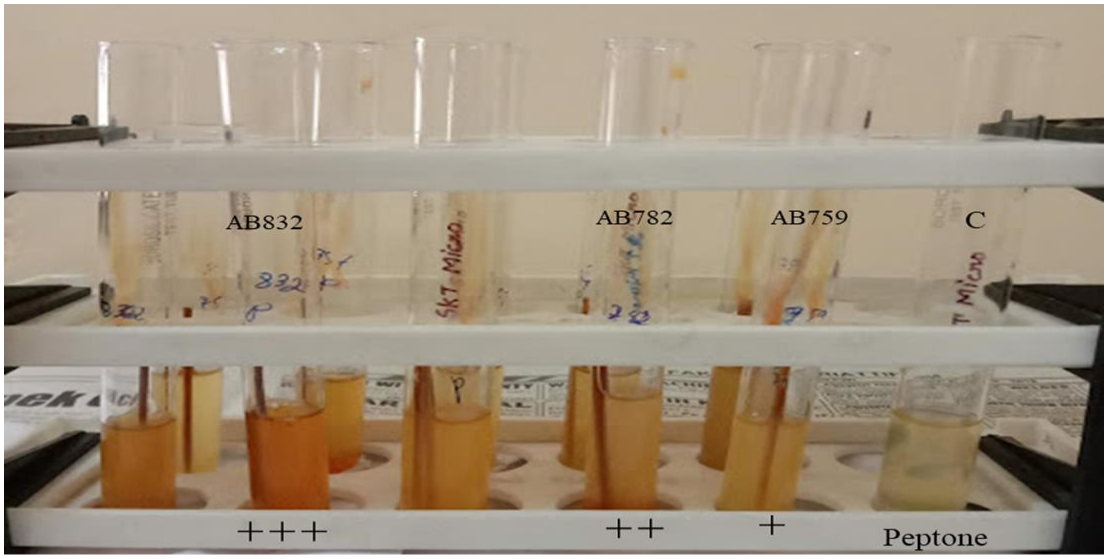



**Fig. 4.5:** Ammonia production of the isolates

### 4.3.3. Siderophore production

The production of siderophore was examined using CAS blue agar assay; 4 strains of actinomycetes (AB757, AB774, AB782, and AB832) were able to produce siderophore. The blue colour of the medium to orange or presence of yellow to light orange halo surrounding the colony indicates the production of siderophore. Strains AB782 and AB832 showed maximum siderophore production followed by strain AB774 and strain AB757 (Table 4.2., fig. 4.6).

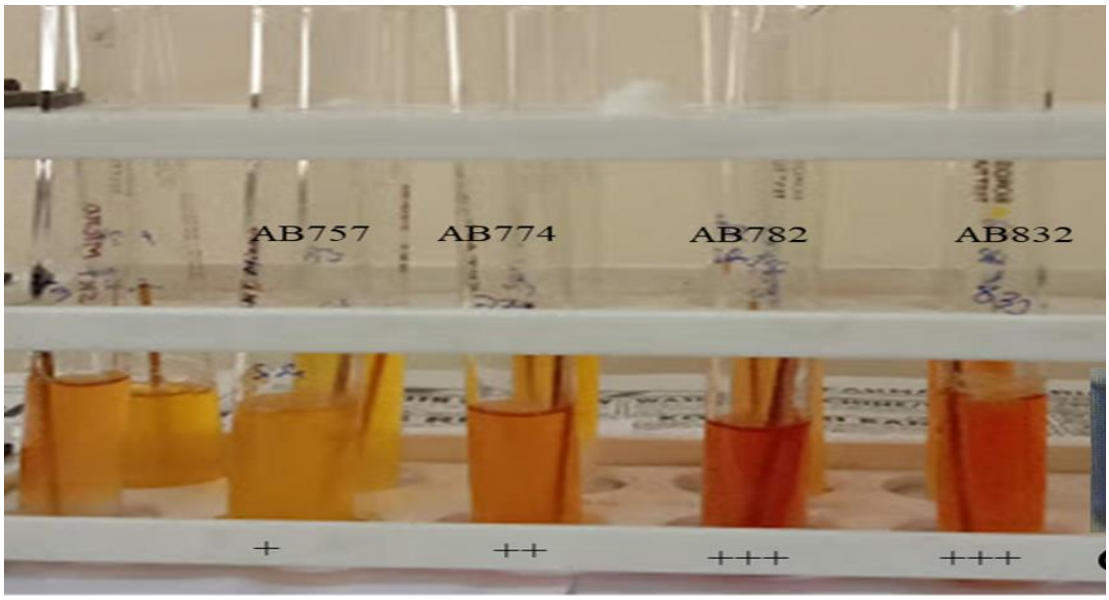



**Fig. 4.6:** Siderophore production of the isolates

### 4.3.4. Indole-3-acetic acid (IAA) production

The isolates were grown on International Streptomyces Project 1 (ISP1) broth containing 0.2% L-tryptophan and incubated at 28°C with continues shaking at 125 rpm for seven days at 28°C. Cultures were centrifuged at 11,000 rpm for 15 min. One millimeter of the supernatant was mixed with 2 ml of the Salkowski reagent. The IAA production was observed as the development of a pink to red colour. Out of 35, 5 isolates (AB757, AB759, AB774, AB782 and AB832) were showed positive for IAA production. Highest IAA production ability was recorded in strains AB774 and AB832 followed by strains AB757 and AB759 (Table 4.2., fig. 4.7).

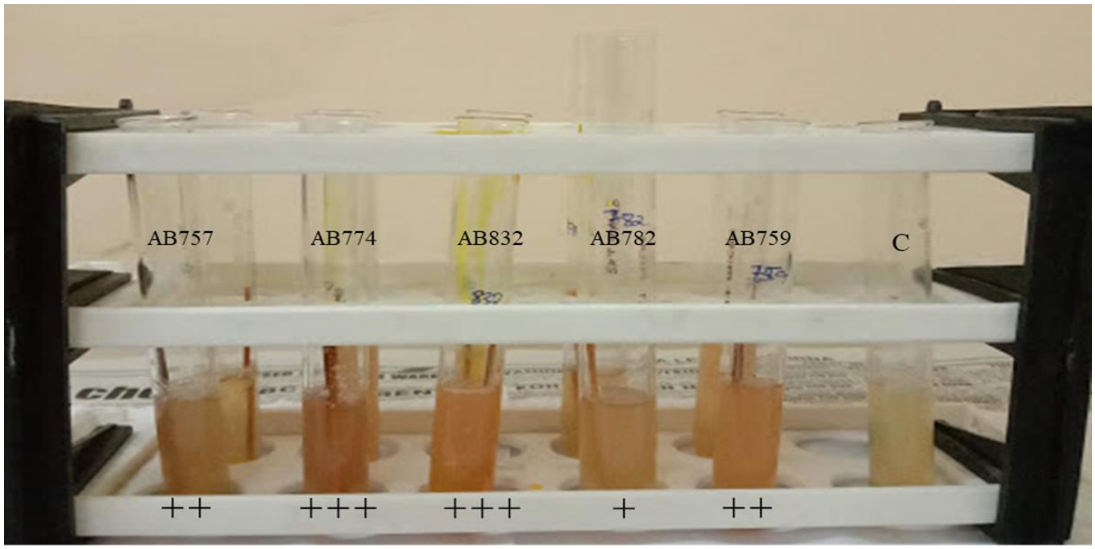



**Fig. 4.7:** IAA production of the isolates

### 4.3.5. Nitrogen fixation

The culture were inoculated into sterilized N free medium, under aseptic conditions and incubated at 28 ± 2°C. Out of total isolates, strain AB832 showed turning from yellow to green colour were confirmed to have the capacity of fixing atmospheric nitrogen (Table 4.2., fig. 4.8).

### 4.3.6. Amylase production

Among the total isolates, only 2 isolates (AB782 and AB832) were found to be the amylase producers in starch agar. Both the strains AB782 and AB832 were able to produce maximum amylase production (Table 4.2., fig. 4.8).

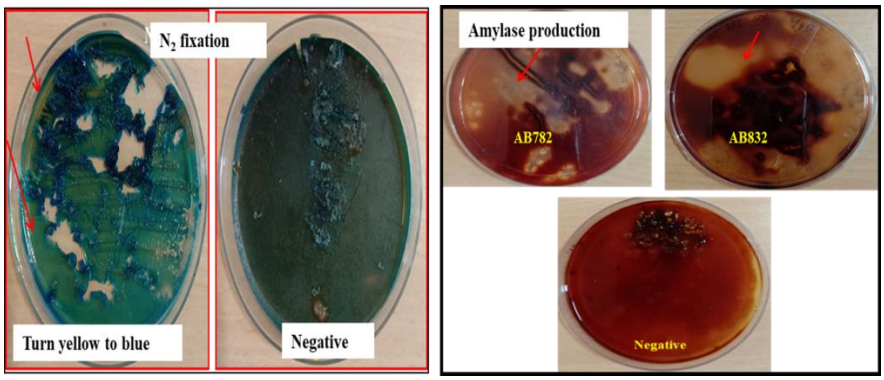



**Fig. 4.8:** Nitrogen fixation ability of AB832 isolate and amylase production isolates AB832 and AB782

### 4.3.7. Catalase test

A drop of 3% $H_2O_2$ in the culture tube observe for the evolution of oxygen bubbles. Out of total isolates, 8 isolates (AB761, AB782, AB832, AB757, AB770, AB828, AB726 and AB759) were observed copious bubbles produced (Table 4.2., fig. 4.9).

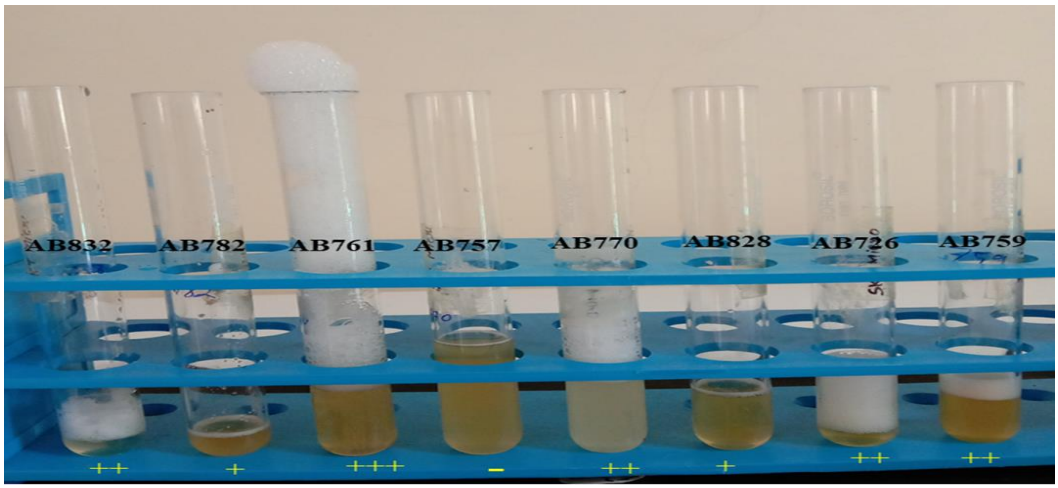



**Fig. 4.9:** Catalase production of the isolates

**Table 4.2:** *In-vitro* screening of the isolates for PGP activities (+, ++, +++ & – indicates to low, medium, high and no production)

| Sl. no. | Isolate code | Organism | Phosphate | IAA | Amm-onia | Side-rophore | N2 fixation | Amyl-ase | Cata-lase |
|---|---|---|---|---|---|---|---|---|---|
| 1. | AB757 | *Streptomyces venezuelae* | + | ++ | – | + | – | – | + |
| 2. | AB726 | *Micromonospora auratinigra* | + | – | – | – | – | – | ++ |
| 3. | AB761 | *Streptomyces avellaneus* | + | | | | | | +++ |
| 4. | AB770 | *Streptomyces seoulensis* | + | – | – | – | – | – | ++ |
| 5. | AB774 | *Streptomyces scabiei* | + | +++ | | ++ | | | |
| 6. | AB782 | *Streptomyces vinaceus* | + | + | ++ | +++ | | +++ | + |
| 7. | AB784 | *Streptomyces scabiei* | + | – | – | – | – | – | – |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 8. | AB788 | *Streptomyces* sp. | + | – | – | – | – | – | – |
| 9. | AB832 | *Streptomyces* sp. | + | +++ | +++ | +++ | +++ | +++ | ++ |
| 10. | AB828 | *Streptomyces mirabilis* | + | – | – | – | – | – | + |
| 11. | AB759 | *Streptomyces venezuelae* | + | ++ | – | – | – | – | ++ |

### 4.4. *In-vivo* assessment of the selected rhizospheric actinomycetes

Strain AB832 showed positive PGP activities for all the tested PGP traits such as phosphate solubilization, nitrogen fixation, IAA, siderophore, ammonia, amylase, catalase production which was selected as the best isolates among the 11 potential PGP actinomycetes for *in-vivo* assessment. Selected strain was evaluated for growth promoting ability in bean and maize plants. The seeds of these plants were collected from the local *jhum* cultivators and they were sown in *jhum* soil under control condition. The germination percentage and plant growth promoting activity was studied by growing the 2 sets of bean and maize seeds for 20 days. Then the germination percentage and plant growth promotion activity was measured and compared with the control.

#### 4.4.1. Seed germination assay

Both bean and maize plants, each ninety six seeds inoculated with suspension of isolate AB832 ($10^{-6}$ $ml^{-1}$). Out of 96 seeds, 72 (75%) bean seeds and 60 (62.5%) maize seeds were germinate (Fig.4.10).

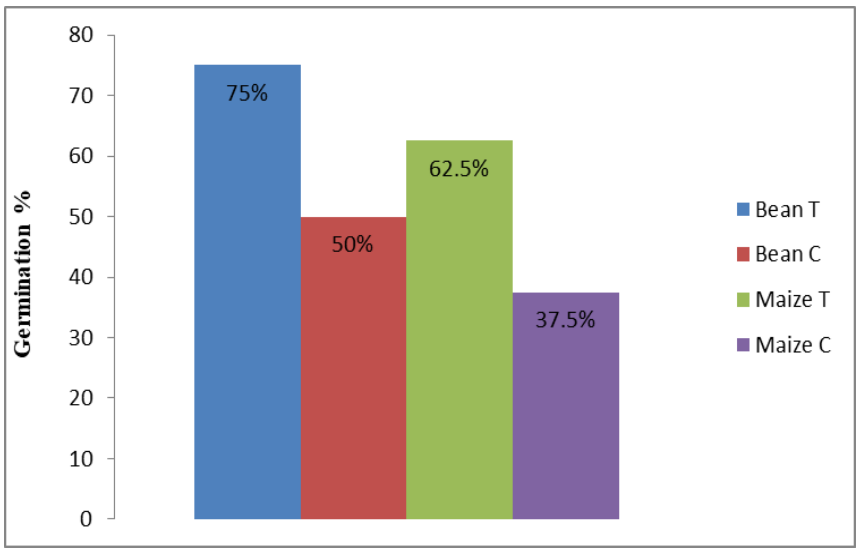



**Fig.4.10:** Change in germination percentage (%) with isolate AB832 compared with the control. Germination percentage of bean 72 (75%) number of seeds (1.42±0.39) out of 96 seeds under the treatment (T) compared with control 48 (50%) number of seeds (1.28±0.38) and maize 60 (62.5%) number of seeds (1±0.33) out of 96 seeds under the treatment (T) compared with control (C) 36 (37.5%) number of seeds (1.26±0.4)

### 4.4.2. Effect of AB832 on plant growth promotion of beans

The effects of isolate AB832 on growth promotion activity in bean plants were demonstrated under mist chamber conditions.

At 20 days after treatment, significant increase in fresh weight (0.406±0.128), surface area (413.6±130.7) and average length (439.7±139) of the shoots were observed in plants treated with AB832 isolate (T) as compared to control (C). However, there was no significant difference in average diameter and dry weight of the shoots between T and C for the bean plants. In contrast, surface area (225±71.4), fresh (0.015±0.004) and dry weight (0.014±0.004) and the average length (349.2±110.4) of the roots were recorded to be lower in T than C (Fig. 4.12). Images of shoot and root for the bean plants with treatment AB832 isolate and without treatment were analysed by the software WinRhizo2012b and is shown in Fig. 4.11, 12 & 13.

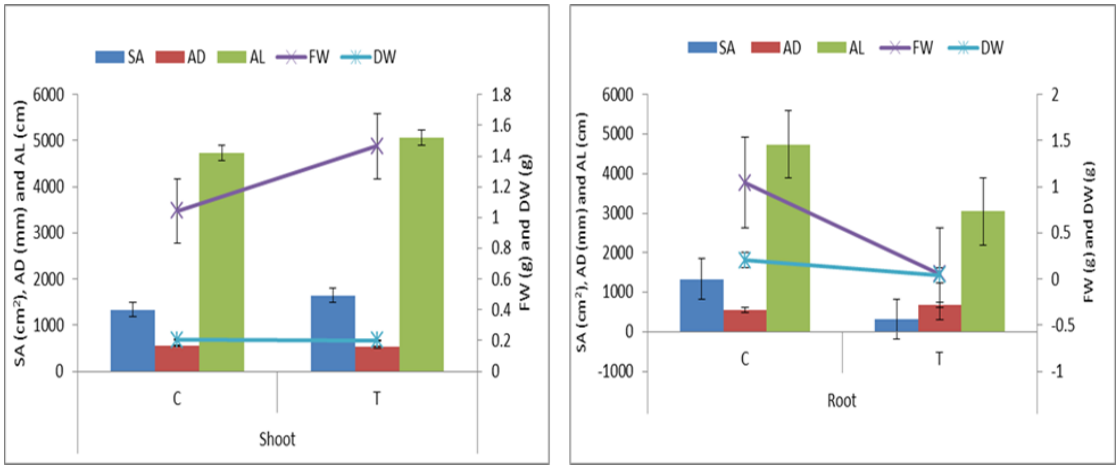



**Fig.4.11:** *In-vivo* plant growth promotion assay in bean plant. Average surface area (SA) (cm2), average diameter (AD) (mm), average length (AL) (cm), fresh weight (FW) (g), dry weight (DW) (g). Evaluation was made after 20 days of growth. Bars representing mean±SE of 10 replicates (10 plants). Data were statistically analysed using a one-way ANOVA and least significant difference (LSD) tests at $p \leq 0.05$ ($LSD_{0.05} = 2515.1$). Highly significant positive correlation between treated and control plants for shoot (0.99869953=1) and root (0.97021169=1) morphology

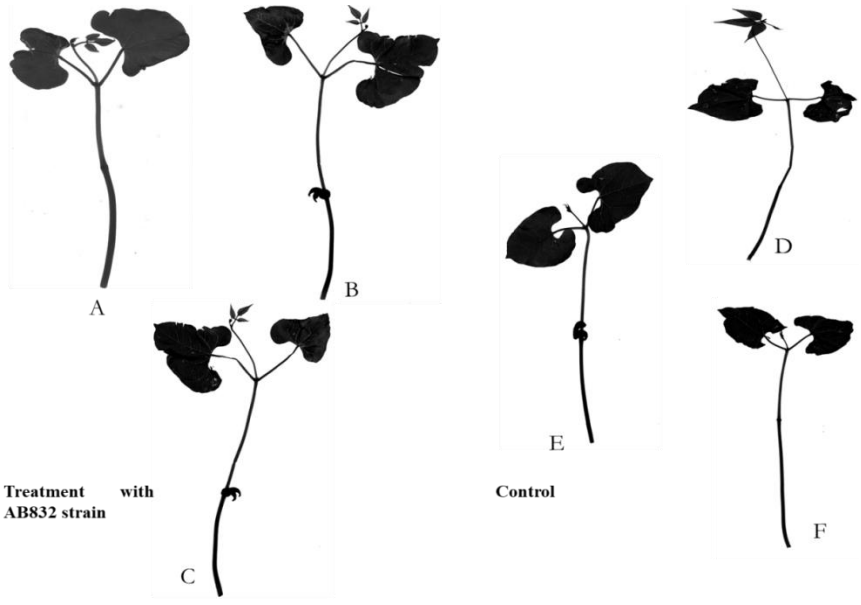



**Fig.4.12:** Effect of AB832 strain on bean shoot images analysed by winRhizo2012b software. A, B, C representatives of treatment and D, E, F as control

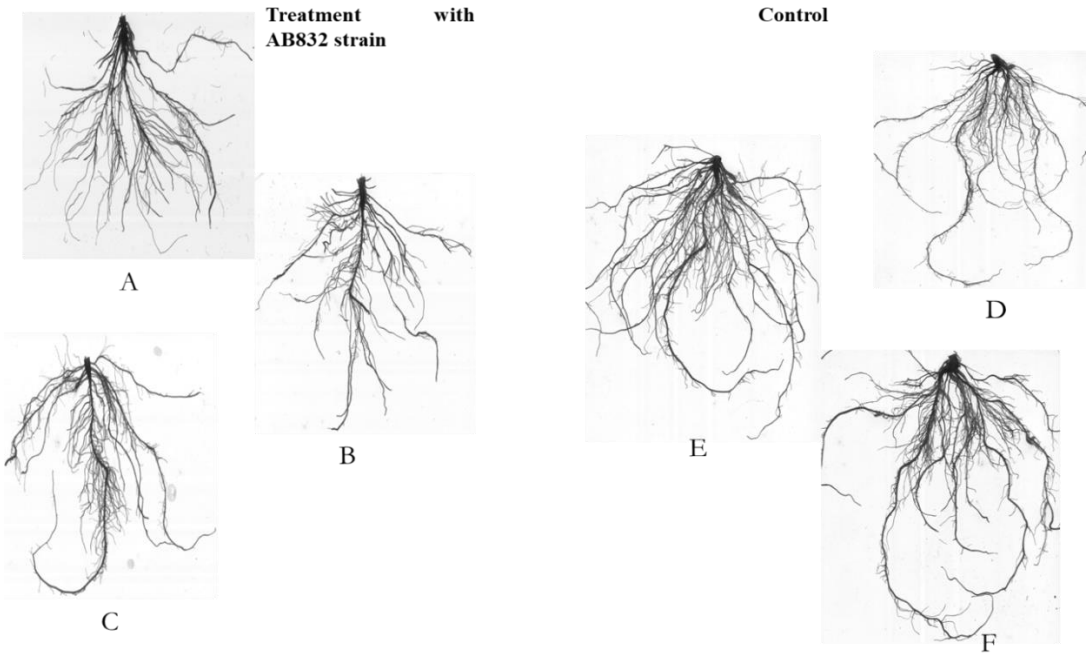



**Fig. 4.13:** Effect of isolate AB832 strain on bean root images analysed by winRhizo2012 software. A, B, C representatives of treatment and D, E, F as control

### 4.4.3. Effect of AB832 on plant growth promotion of maize

The plant growth promotion activity of isolate AB832 inoculated in maize plants under mist chamber conditions of 20 days showed variable growth parameters after 20 days (Fig.7). Treatment with isolate AB832 (T) resulted in increase in surface area (565.5±252.9), average diameter (505±225.8) and fresh (0.397±0.177) and dry weights (0.030±0.013) of the shoots when compared to control (C) plants. However, no change was observed in average length (1153.7±515.9) of the shoots of T as compared to C. Significantly enhanced growth in surface area (237.3±75), average diameter (210.1±66.4) and average length (211.8±67) and dry weight (0.039±0.01) of the roots were observed with T over C. However, fresh weight (0.197±0.062) of the roots was found to increase in T (Fig.4.14). WinRhizo2012b-analysed images of shoots and roots of the control maize plants and the maize plants treated with the isolate AB832 are shown in Fig.4.14, 15 &16.

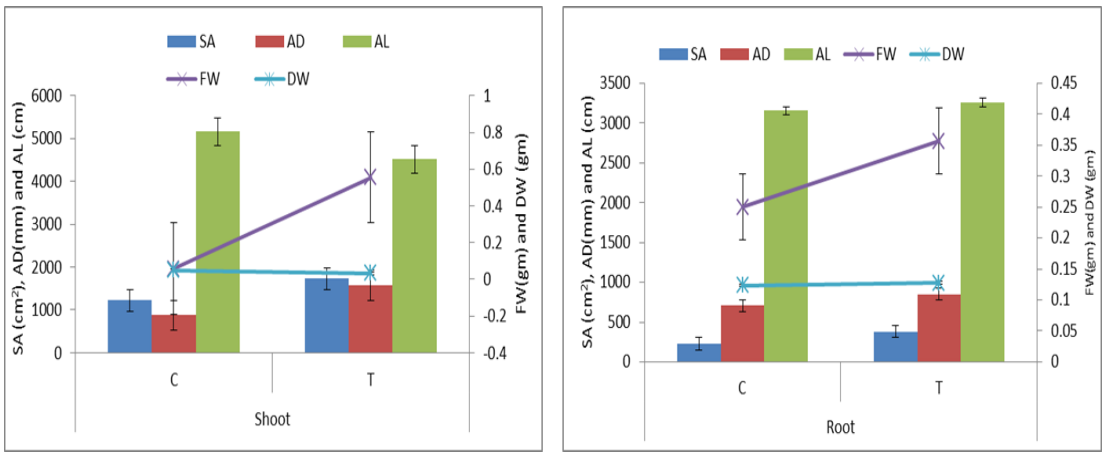



**Fig.4.14:** *In-vivo* plant growth promotion assay in maize plant. Average surface area (SA) (cm2), average diameter (AD) (mm), average length (AL) (cm), fresh weight (FW) (g), dry weight (DW) (g). Evaluation was made after 20 days of growth. Bars representing mean±SE of 5 replicates (5 plants). Data were statistically analysed using a one way ANOVA and least significant difference (LSD) tests at $p \leq 0.05$ ($LSD_{0.05} = 1618.2$) were performed. Highly significant positive correlation between treatment and control of shoot (0.976179=1) and root (0.99857=1) was observed

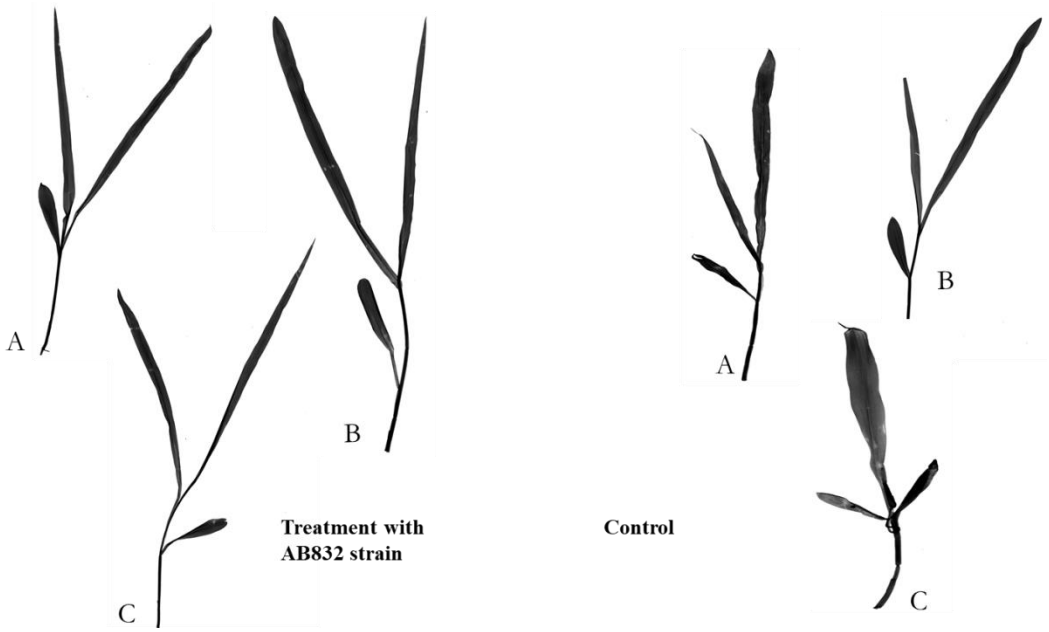



**Fig.4.15:** Effect of AB832 strain on maize shoot images anlaysed by winRhizo2012b software. A, B, C representatives of treatment and D, E, F as control

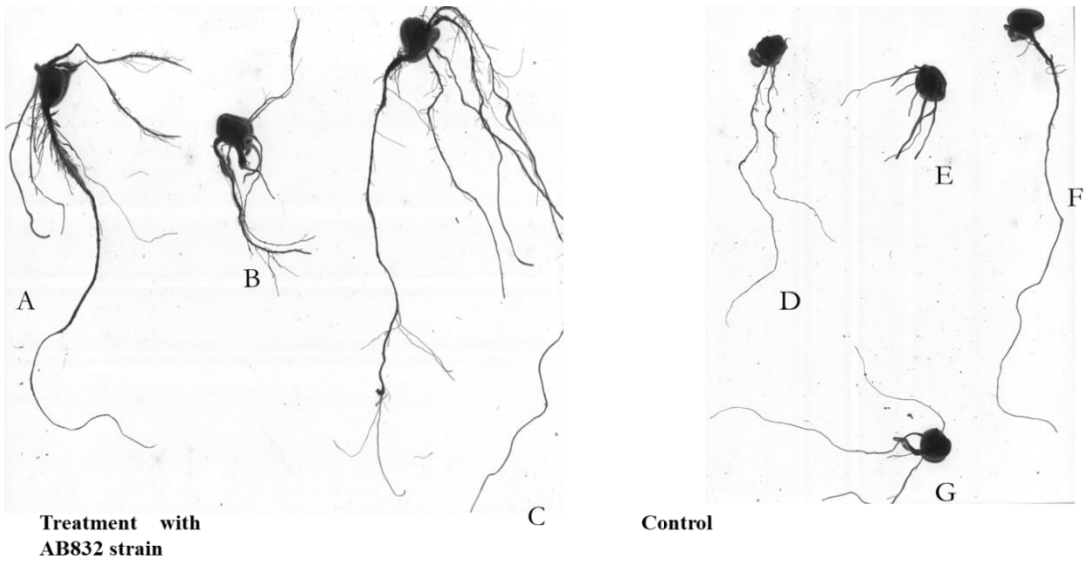



**Fig. 4.16:** Effect of AB832 strain on maize root images analysed by winRhizo2012 b software. A, B, C representatives of treatment and D, E, F as control

### 4.4.4. Effect of AB832 on rhizospheric soil properties

The results of soil analysis showed considerable variations in soil properties between treated and control soils of bean and maize crops. Variations were statistically significant in treated rhizospheric soils of bean and maize in relation to control. In bean, significantly lower amount of available nitrogen (N), potassium (K), and pH was recorded in treatment of AB832 isolate. Further, higher soil organic carbon was recorded in treatment with AB832 isolate compared to control. There was no significant difference in phosphorus (P) content between treatment of AB832 isolate and control. Whereas, in maize, statistically significant increase in the pH, N, K, soil organic carbon (SOC) was recorded in treatment with AB832 isolate compared to control. There was no significant difference in available phosphorus between treatment with AB832 isolate and control (Fig. 4.17).

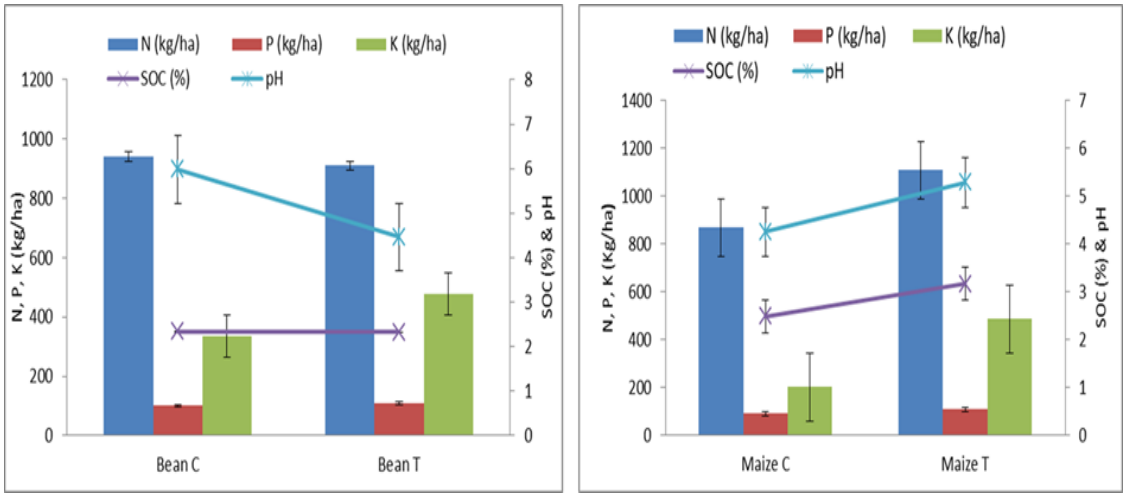



**Fig.4.17:** Effect of isolate AB832 on rhizopsheric soil properties of bean and maize under control condition. Bars representing mean±1SE of 3 replicates (3 replicates of soil sample). Data were statically analysed using a one way ANOVA and least significant difference (LSD) tests at $p \leq 0.05$ ($LSD_{0.05} = 546.8$). Showed highly correlated between treatment bean and control bean (0.985195738=1), between treatment maize and control maize (0.975959392=1)

### 4.4.5. Analysis of rhizospheric soil properties of the study area

The rhizospheric soil properties showed variations in N, P and K content in two *jhum* cultivation sites i.e. Tanhril and Reiek. P and K were significantly higher in Reiek as compared to Tanhril. N was lower in Reiek compared to Tanhril. No significant difference in SOC and pH were recorded in Tanhril and Reiek (Fig. 4.18).

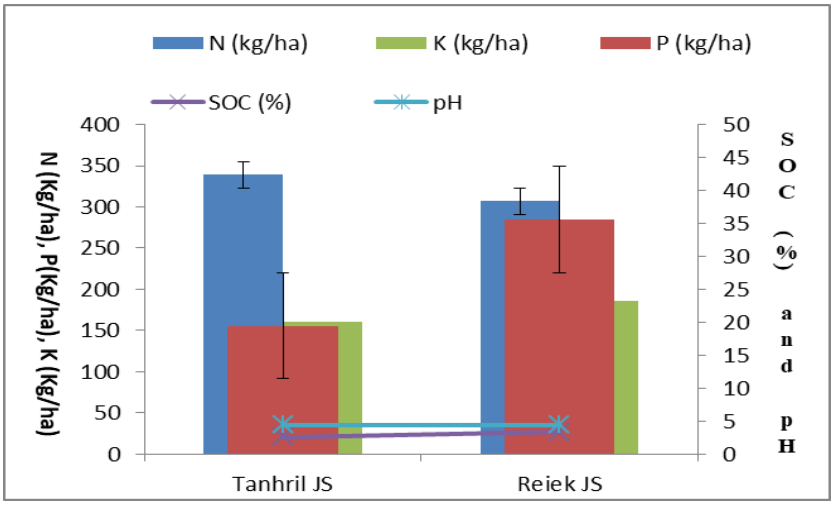



**Fig.4.18:** Data were statically analysed using one way ANOVA and least significant difference (LSD) tests at $p \leq 0.05$ ($LSD_{0.05} = 205$) for rhizospheric soil of *jhum* in Reiek and Tanhril

## 4.5. Identification of PGP potential actinomycetes strains using 16S rRNA gene sequencing

A fragment of the 16S-rRNA gene of eleven isolates, which exhibited significant PGP activity, was sequenced. Based on the similarity percentage (99-100%) of 16S rRNA gene sequences of the isolated organisms it was found that 10 isolates belonged to *Streptomyces* and one isolate belonged to *Micromonospora* genera. Obtained sequence revealed that majority of the isolates belonged to family Streptomycetaceae which comprised 90% of the total isolates, followed by family Micromonosporaceae (9%). The sequences were deposited in NCBI GenBank (Accession number MN326854 -MN326864) (Table 4.3).

**Table 4.3:** 16S rRNA gene sequencing of 11 rhizosphere actinomycetes

| Sl. no. | **Isolate no.** | **NCBI GeneBank accession no.** | **Closest species** | **Similarity** |
|---|---|---|---|---|
| 1. | AB757 | MN326854 | *Streptomyces venezuelae* | 99% |
| 2. | AB726 | MN326855 | *Micromonospora auratinigra* | 99% |
| 3. | AB761 | MN326856 | *Streptomyces avellaneus* | 99% |

| | | | | |
|---|---|---|---|---|
| 4. | AB770 | MN326857 | *Streptomyces seoulensis* | 99% |
| 5. | AB774 | MN326858 | *Streptomyces scabiei* | 99% |
| 6. | AB782 | MN326859 | *Streptomyces vinaceus* | 99% |
| 7. | AB759 | MN326860 | *Streptomyces venezuelae* | 99% |
| 8. | AB828 | MN326861 | *Streptomyces mirabilis* | 100% |
| 9. | AB784 | MN326862 | *Streptomyces scabiei* | 100% |
| 10. | AB832 | MN326863 | *Streptomyces* sp. | 99% |
| 11. | AB788 | MN326864 | *Streptomyces* sp. | 99% |

### 4.6. Phylogenetic analysis of PGP potential actinomycetes strains

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-1090.8260) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and Bio NJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1861)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 425 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Fig. 4.19).

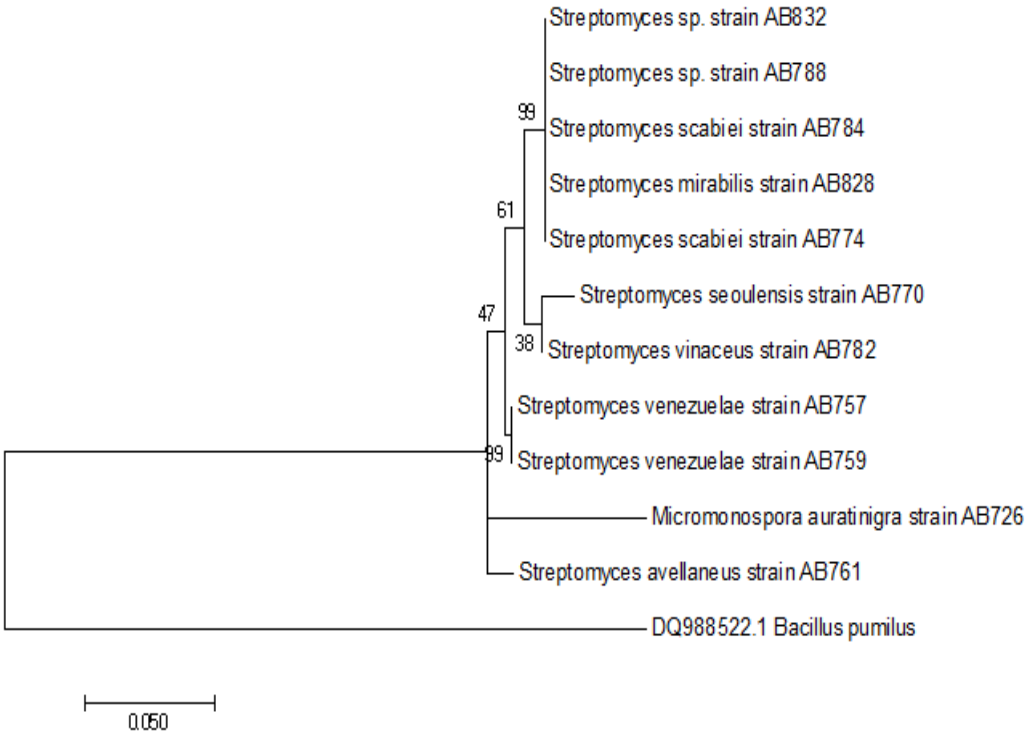



**Fig.4.19:** Phylogenetic relationships of the 11 rhizosphere actinomycetes

# Chapter 5

# Discussion

## 5.1. Problems of soil fertility in shifting cultivation and the role of actinomycetes

Shifting cultivation or slash and burn agriculture, locally known as *jhum*, is a dominant form of agricultural practice in Northeast India, which is badly hampered by the decreasing soil fertility and crop productivity due to reduced fallow length to <5 years compared from earlier 20-30 years. As a result, majority of farmers associated with this practice are facing problems of food security. There various scientific interventions have been made by the State Government to improve the systems but none of them were able to solve the problem of this practice either because of one or another region. Developing bio fertilizer with the help of recent biotechnological tools using indigenous soil microbes especially actinomycetes and their inoculation in the crop plants would be one of the important efforts to boost the soil fertility and crop productivity in the shifting cultivation in the region. Therefore, this study has isolated indigenous actinomycetes from different crops of shifting cultivation of Mizoram and to inoculate them into crop plants to assess their effects on soil fertility and crop production, and ultimately on the livelihood of the *jhumias*.

## 5.2. Actinomycetes association with plant rhizosphere and their ecological importance

Actinomycetes are ubiquitous in nature (Chamikara, 2016) and widely distributed as free-living saprophytes forming thread-like filaments in the soil (Jeffrey, 2008; Salim *et al*., 2017). Actinomycetes are abundant in soil and play a role in decomposing toughest things like starches, cellulose and proteins to break down (Tiwari *et al*., 2019). Actinomycetes hold a prime attention in the production of a large number of bioactive secondary metabolites (Berdy, 2012) such as antibiotics (Strohl, 2004), antitumor agents Cragg and Newman, 2005), immunosuppressive agents (Mann,

2001), enzymes (Oldfield *et al*., 1998; Pecznska-Czoch and Mordarski, 1988). Secondary metabolites obtained from actinomycetales provide a potential source of many novel compounds with antibacterial, antitumour, antifungal, antiviral, antiparasitic and other properties (Solecker *et al*., 2012).

Actinomycetes have been recognized as one of the major components of plant rhizosphere which is beneficial in soil nutrient cycling and promotes plants growth-promotion (PGP) (Halder *et al*., 1991; Elliot and Lynch, 1995; Merzaeva and Shirokikh, 2006). The rhizosphere is a hot-spot of high microbial activity which can serve as an important source for the plant growth and development (Sorenson, 1997). Rhizosphere microorganisms are very likely to influence the soil properties which in turn affect crop plants. Shifting cultivation offers a unique opportunity to study the role of actinomycetes in the rhizsophere of traditional crops of shifting cultivation practice. In view of the agro active importance of such organisms, isolation and plant growth-promoting potential characterization of native microbes from major crops under *jhum* fields of Mizoram, Northeast India has become particularly essential.

### 5.3. Isolation of rhizospheric actinomycetes from crop plants of shifting cultivation

In the present study, total 35 rhizospheric actinomycetes were isolated from the rhizosphere of major crops under shifting cultivation of Mizoram, Northeast, and India. They have been identified with respect to morphological characteristics (Table 4.1., fig. 4.3 & 4.4). Most actinomycetal isolates showed mycelium of varying colours, sticky-hard, hairy or smooth colonies, slow growth nature, production of pigments and also produced earthy or musty odour. Similar observations were noted by Araujo, 1998; Sreevidya *et al*., 2016; Raut and Kulkarni, 2018). Microscopically, it was identified that the spore chains were straight or flexuous forms, hooks, open loops, coils or hairy structures, morphology of the spore chains varied depending on the actinomycetes species (Fig.4.4). According to Taddei *et al*., 2006, our results showed a confirmatory identification to genus level. In the present investigation, total isolates was appeared close to *Streptomyces* species. Araujo *et al*., 1998 suggested that the distribution of *Streptomyces* spp. in various ecosystems is due to their ability to adaptation to extensive range of environmental conditions and hence, they may

develop resistant to plant pathogens and potential to produce plant growth-promoting properties. Based on media employed, 12 isolates (34.2%) were able to grow in CSM followed by 11 isolates (31.4%) in SCA media, 8 isolates (22.8%) were obtained from ISP2 media and 4 isolates (11.4%) from IM8 media (Fig. 4.2). Similar results were also reported that rhizosphere actinomycetes are most frequently isolated from the CSM followed by SCA and ISP2 medium (Sengupta *et al*., 2015). Total 35 rhizospheric actinomycetes were obtained in the present study where 3 (8.5%) actinomycetes were isolated from *jhum* cultivation of Tanhril and 32 (91.4%) from *jhum* cultivation of Reiek (Fig. 4.1). The diversity of actinomycetes in the rhizospheric soil was isolated more from Reiek *jhum* soil than Tanhril *jhum* soil. The abundance of *Streptomyces* species have been proven maximum in Reiek *jhum* soil while one isolates *Micromonospora* species which is from other genera and family were isolated from Tanhril *jhum* soil. This could be the result of many factors including the status of soil nutrients following burning, which reflected changes in microbial communities of actinomycetes. The edaphic factors include soil pH and available soil nutrients that are important to the actinomycetes. Rhizospheric soil phosphorus, potassium and soil organic carbon increased greatly in Reiek *jhum* soil whereas nitrogen was high in Tanhril *jhum* soil (Fig.4.18). This means that phosphorus and potassium may greatly affects microbial communities of actinomycetes. These results suggested that most rhizospheric actinomycetes particularly *Streptomyces* species are poorly adapted to survive the periods of low phosphorus and potassium in the rhizospheric soil. Increase in nitrogen may favours the survival of other genera particularly *Micromonospora* species. The rhizospheric soil of Reiek *jhum* site was more acidic than Tanhril *jhum* soil (Fig.4.18). This observation was further confirmed that most of the *Streptomyces* species have the capacity to survive in the high soil pH or increase soil pH may be suitable to actinomycetes, *Streptomyces* species. Previous researcher, Adeniyi, 2010 suggested that this could be as a result of the burning vegetation may release a pulse of nutrients to the soil and ash that increases the soil pH.

## 5.4. *In-vitro* screening of plant growth-promoting properties of the isolates

The total 35 isolates were characterized morphologically and were subjected to a comprehensive *in-vitro* screening for various plant growth promoting (PGP) traits. Out of total isolates, 11 (31.4%) of the isolates screened were found to be the promising PGP rhizospheric actinomycetes (Table 4.2), among them, the ten isolates belongs to genus *Streptomyces* and one isolate belongs to *Micromonospora*. These 11 isolates showed PGP positive at least one tested PGP traits and were selected for further studies. The most frequently occur rhizospheric actinomycetes isolates from major crop plants in our study was genus *Streptomyces* and similarly in previous studies reported maximum isolates belong to genus *Streptomyces* (Anwar *et al*., 2016; Sreevidya *et al*., 2016). This suggests that the strains of *Streptomyces* are able to establish relationship easily in the variety of plant rhizosphere. Our results demonstrated that rhizospheric actinomycetes associated with the major crop plants promote plant growth through production of plant growth regulators (i.e. IAA, siderophore, ammonia, phosphate solubilization, nitrogen fixation, and catalase and amylase production). Many researchers have found that rhizosphere actinomycetes are a group to be the most potential candidates of biofertilizer agents. Actinomycetes genera have been widely developed for increasing agricultural crops productivity including *Actinoplanes*, *Streptomyces* and *Micromonospora* and among them, *Streptomyces* have been reported as the most explored species in respect to the plant growth promoting activity. Previous researchers, Jog *et al*., 2012 reported that two *Streptomyces* sp. isolated from the wheat rhizosphere attributed with high plant growth promoting activities and chitinase-phytase productions could significantly promote wheat growth. Sousa *et al*. (2008) also reported three *Streptomyces* sp. which was capable in producing siderophores, solubilizing phosphate and producing phytohormones IAA, as potential PGPR agents. Fifty-three rhizospheric actinomycetes isolates was isolated from soybean. Among 53 isolates, 18 (34%) isolates were showed production of IAA in range of 2.08 ppm to 16.70 ppm. and 5 isolates were able to grow on nitrogen-free medium and solubilize phosphate. Based on 16S rRNA gene sequencing analysis, isolates were highly similar with *Streptomyces* genera (Wahyudi *et al*., 2019).

### 5.4.1. Phosphate solubilization

It was reported that actinomycetes play an important role in acidification of external medium by production of low molecular weight organic acids like gluconic acid (Chen *et al*., 2006). The most common and effective acids involved in the inorganic phosphate solubilzation are guconi acid, lactic acid, malic acid, succinic acid, formic acid, citric acid, malonic acid, and tartaric acid (Liu *et al*., 2014). Moreover, solubilization of the inorganic phosphate is a promising point for the selection of bacteria accomplished of increasing accessible phosphorus in the rhizosphere (Dutta *et al*., 2015). These microbes can be significant source as PGPR in the biofertilization of crops. These bacteria secrete different types of organic acids (e.g., carboxylic acid) thus lowering the pH in the rhizosphere and consequently release the bound forms of phosphate like $Ca_3$ $(PO_4)_2$ in the calcareous soils. Utilization of these microorganisms in the agricultural program as environment-friendly biofertilizer may help to reduce the use of synthetic phosphatic fertilizers. Phosphorus biofertilizers could increase the availability of accumulated phosphate, increase the efficiency of biological nitrogen fixation and render availability of Fe, Zn, etc., through production of plant growth promoting substances. The phosphate solubilization activity was detected to 11 (31.4%) isolates in the present study (Table 4.2). These results are in agreement with Hamdali *et al*., 2008, who reported that *Streptomyces* species and *Micromonospora* species were able to solubilize inorganic phosphate.

### 5.4.2. Ammonia production

Present study detected two (5.7%) isolates AB759 and AB832 of the rhizospheric actinomycetes having potential to produce ammonia (Table 4.2, fig.4.5). Marques *et al*. (2010) suggested that ammonia production also play an important role in plant growth by supplying nitrogen to the host plant and helps in increase root and shoots growth, consequently increase plant biomass production. Production of ammonia serves as a prompting factor for the virulence of opportunistic plant pathogens and also play an important role in suppression of plant disease (Anwar *et al*., 2016). In this study, ammonia producing isolates were belonging to the genus *Streptomyces*.

Similarly, Passari *et al*., 2015b reported that *Streptomyces* strain as a potent ammonia producer.

### 5.4.3. Siderophore production

This study detected four (11.4%) isolates namely AB757, AB774, AB782, and AB832 belonging to *Streptomyces* sp. which have shown siderophore production ability (Table 4.2, fig.4.6). Siderophore production is one of the essential factors for the growth of plant (Tan *et al*., 2009). Siderophores are referred to as microbial Fe-chelating low molecular weight compounds. This compound after chelating iron ($Fe_3^+$) makes the soil iron ($Fe_3^+$) poor for other soil microbes and consequently inhibits the activity of competitive microbes in which iron availability is limiting factor (Siddiqui, 2005; Cao *et al*., 2005). The presence of siderophore producing actinomycetes in the rhizosphere that stimulates plant growth and productivity of crops. Further, siderophores ability to form various chemical structures and form a family of at least 500 different compounds. Such as antibiotics (i.e., albomycins, ferrimycins, danomycins, salmycins, and tetracyclines) can bind Fe and some siderophores showed diverse biological activities (Wang *et al*., 2014). The actinomycetes belong to genus *Streptomyces* sp. have high resistant to heavy metals and the role of the elicited siderophores in promoting plant growth under iron and nickel stress are described by Dimkpa *et al*., 2008; Schutze *et al*., 2015.

### 5.4.4. IAA production

In this study, 14.2% of the isolates were positive for IAA production. Among them, isolates AB774 and AB832 maximum IAA production ability followed by strains AB757 and AB759 (Table 4.2., fig.4.7). The IAA production of actinomycetes was in accordance with Khamna *et al*., 2009; Verma *et al*., 2012. The IAA synthesized by the actinomycetes was responsible for the development of adventitious root volume by which roots help plant to take nutrients and increased root exudates and in turn influence bacteria (Alla *et al*., 2013). The rhizospheric actinomycetes associated with plant produce IAA that plays an important role in host plant development and growth.

### 5.4.5. Nitrogen fixation

Nitrogen is the most vital and limiting nutrient for plant growth and productivity. Nitrogen limits plant growth in many terrestrial ecosystems of the world. Bulk of nitrogen is present in the atmosphere as di nitrogen, which intern gas and cannot be used by the living organisms from the metabolism. Small microorganisms found in the soil are capable of fixing atmospheric and nitrogen in the soil. Nitrogen fixation is the process whereby atmospheric nitrogen is converted to ammonia with the aid of an enzyme called nitrogenase (Kim and Rees, 1994). Interaction of plants and nitrogen fixers made available nitrogen such as ammonia and nitrate (Wagner, 2011). Symbiotic bacteria that can process through root nodules to sequester atmospheric nitrogen in the form of ammonia, a form of nitrogen that can be assimilated into organic components including proteins and nucleic acids (Unkovich *et al*., 2008; Pankievicz *et al*., 2015). In the present study, only one isolate AB832 had ability to fix atmospheric nitrogen as this strain has ability to grow on nitrogen-deficient media (Table 4.2, fig. 4.8). Present result open doors to the researcher advances in microbial inoculants to explore diverse ecosystem, use friendly biological resource for sustainable management of agriculture. Furthermore, nitrogen fixing microorganisms signifies an economically positive and environmentally sound alternative to chemical fertilizers (Khalid *et al*., 2004).

### 5.4.6. Amylase and catalase production

Microbial amylases are considered as important group of enzymes that applied in many industries that hydrolyse starch into high fructose, glucose and maltose syrups and can be categorized into endoamylases and exoamylases. Actinomycetes are one of the most diverse groups of microorganisms that are well known for their metabolic versality. Amylases from *Streptomyces* sp. play an important role in biotechnological applications in different industries and having approximately 25% of demand in the world enzyme market (Mukhtar *et al*., 2017). Among the total amylase screening, two (5.7%) isolates AB782 and AB832 showed amylase production with the zone formation by hydrolyzing starch agar medium (Table 4.2, fig.4.8 & 9). These results indicated that amylase producer actinomycetes can be isolated from the crops under shifting cultivation. It is also demonstrated that rhizospheric actinomycetes

particularly *Streptomyces* have potential to secrete amylase enzyme. The α amylase starch degrading amylolytic enzymes is of great significance in biotechnological applications such as food industry, fermentation and textile to paper industries (Pandey *et al*., 2000). Ramesh and Mathivanan, (2009) reported actinomycetes having ability to produce industrial enzymes such as lipase, amylase, cellulase, caseinase and gelatinase.

Out of total isolates, 4 (11.4%) isolates AB761, AB782, AB832 and AB759 were able to produce catalase (Table 4.2, fig.4.9). Catalase is another dismutase enzyme; it catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS). Catalase is responsible at degrading high concentrations of hydrogen peroxide, such as might be found in peroxisomes, the subcellular organelle where most catalase is localized. In the present study, all the actinomycetes produced enzymes which are specialized proteins indicating their ability to convert complex molecules into simpler ones and release them into the environment to break down nutrients into smaller forms that helps to make them available to plants for uptake. Amylase and catalase production by rhizospheric actinomycetes could be important plant growth-promoting factors. These microbes producing amylase may play a significant role in decomposition of organic matter, nutrient mineralization, PGP (Lima *et al*., 1998). Therefore, rhizospheric actinomycetes are essential metabolite since they possess a capacity to produce and secrete a variety of extracellular hydrolytic enzymes (Saadoun *et al*., 2007; Tan *et al*., 2009). Actinomycetes have been studied from various plants, plant tissues and rhizospheric soil in which their biological functions may depend on sources from which they are isolated (Sharmin, 2005). Rhizosphere actinomycetes of the major crops of shifting cultivation have shown attractive source able to produce amylase and catalase.

### 5.5. *In-vivo* assessment of the selected rhizospheric actinomycetes

In the present investigation, the effect of *Streptomyces* spp. on PGP including root development was studied under greenhouse condition by pot experiment method. Among the eleven PGP potential actinomycetes, isolate AB832 belong to *Streptomyces* sp. showed positive to all the tested PGP traits such as phosphate

solubilization, ammonia, siderophore, IAA production, nitrogen fixation, and amylase and catalase production. *Streptomyces* sp. (AB832) strain were selected to use in this study, seed germination assay, root development assay, seedling growth and determination of rhizospheric soil properties by using bean and maize seeds inoculated with PGP *Streptomyces* sp. (AB832) strain.

### 5.5.1. Seed germination assay

Rhizospheric *Streptomyces* sp. isolates (AB832) strain was demonstrated to enhance the germination of bean and maize seeds of 20 days. Inoculation of AB832 strain in bean increased the germination percentage to 75% compared to 50% in the untreated, control seeds and in maize increased the germination percentage to 62.5% compared to 37.5% in uninoculated, control seeds (Fig.4.10). This finding is consistent with the result obtained by Rae-Hyun and Song, 2007, who reported that *Rhodopseudomonas* KL9 and *Rhodopseudomonas* BL6 increased the germination percentage of tomato seeds by 31.8% and 7.6%, relative to untreated, control seeds. Differences in the improvement of germination percentage may depend on the level of bacterial colonization in the seed, seed coat properties, and the amount of bacterial substances that can penetrate into the seed (Sturz and Nowak, 2000). This finding is consistent with Lasudee *et al*., 2018, who state that *Streptomyces* thermocarboxydus isolate S3 increased the germination percentage of mung bean seeds (95–98%) which was statistically higher than the control. Other environmental factors may also influence the growth-stimulating properties of some bacteria (Passari *et al*., 2019). For example, *Streptomyces* sp. (AB832) may produce some phosphorus, ammonia, siderophore, nitrogen fixation, phytohormone (IAA) and enzymes (amylase and catalase) that stimulate bean and maize seeds to germinate.

### 5.5.2. Effect on root and shoot development

Plant growth promoting effect of polyamine producing isolate of *Streptomyces griseoluteus* has been demonstrated in bean (Nassar *et al*., 2003). Whereas, there is still lack of study on the effect of other PGP potential *Streptomyces* sp. strain in bean plant growth promotion in shifting cultivation sites. In the present study, the effect of AB832 strains on root and shoot development in bean after 20 days has

significantly enhanced the plant growth by increasing shoot length, surface area of the shoot and fresh weight under treatment compared to control. Our results demonstrated clearly the effect of PGP on bean shoots. Within 20 days there was no increasing in root length, root surface area or fresh weight and dry weight of the root under treatment compared to control. It showed significant increase of root diameter (Fig.4.11, 12 & 13). Thus, the improvement of the shoot and root may be due to the enhancement of PGP properties produced by isolate AB832. The mechanism by which the isolate AB832 enhanced PGP including IAA, siderophore, ammonia, nitrogen fixation, phosphate and enzymes on bean may be the direct stimulation of root diameter. Slow development of root system under treatment may be due to the microbial activity in the initial stages supplying nutrients and quantity of root exudates. In fact, some root exudates is dependent upon the physiological status and species of plants and microorganisms; some of the exudates act as repellents against the microbes while others act as attractants to microbes (Kang *et al*., 2010). Moreover, the growth of plant root systems is controlled by different soil physicochemical characteristics, properties that in turn may adapt and influence roots themselves and thereby regulating and inducing responses in the rhizosphere (Olanrewaju *et al*., 2019). Interestingly, there was a significantly difference of root development between bean and maize with AB832 in the present study. The association of *Streptomyces* sp. and roots may develop complex interdependent relationships, where the effect of PGP strain may depends on the plant root exudates. The quantity and type of root exudates produced from growing roots vary with plant species and age (Uren, 2000). Root exudates serve as a messenger between roots and rhizosphere actinomycetes in the rhizosphere (Walker *et al*., 2003). Root exudates are responsible for interactions between rhizosphere actinomycetes leading to plant growth-promotion and induced defences against plant pathogens. Communication between actinomycetes and plant which both exchange nutrients for survival. There may be various types of relationships can be developed based on the nutrient abundance in the soil and the actinomycetes ability to interact with the host plant (Hassan *et al*., 2019). The mechanisms involved in plant-microbe interactions are complex. It is therefore suggested that more investigations would be required on

these rhizospheric actinomycetes and their interactions with major crop plant to be useful tool for continuous crop production under shifting cultivation.

In maize the development of shoot and root after 20 days, AB832 significantly increased shoot diameter, shoot surface area and fresh weight, relative to un-inoculated control plants. Isolate AB832 exhibited plant growth in maize plants which may be due to its potential to have significant plant growth promoting potential. Inoculated AB832 demonstrated a significant PGP activity in root development of maize. Significantly increase root length, root diameter, root surface area and fresh weight of the maize root after 20 days of plant growth (Fig.4.14, 15 & 16). This suggests that during the interaction of plant with PGP beneficial actinomycetes in the rhizosphere may initiate plant response to increase maize plant growth by releasing higher root exudation in the initial stages of growth. Glick, 2012 suggested that IAA produced by rhizobacterial also able to increases root exudation of the host plant by loosening the root cell wall which in turn helps in the rhizbacterial colonization and growth. Ammonia production also plays an important role by the accumulation of nitrogen in plant development of root and shoots (Marques *et al*., 2010). Phosphate solubilization activity of isolate AB832, which may responsible for production of organic acids (Ahmad *et al*., 2008). Isolate AB832 possess amylase and catalase properties, these enzymes are responsible for biochemical reaction. All the biochemical transformations in soil are dependent on, or related to the presence of enzymes. Siderophore is an important for nutrient uptake and it helps in developing growth of the plant. The Streptomyces strains have been reported by previous researchers for its PGP potential (Nassar *et al*., 2003; El-Tarabily, 2008; Gopalakrishnan *et al*., 2011b).

### 5.5.3. Effect on rhizospheric soil properties

In the present study, bean and maize were grown with AB832 isolate under shifting cultivation soil for 20 days in control condition. Isolate AB832 were able to alter the rhizospheric soil properties of bean and maize (Fig. 4.17). Interactions between growing plant roots and soil induce changes in the soil rhizosphere that differ from bulk soil (Wang and Zabowski, 1998; Makoi *et al*., 2014). These changes in the rhizosphere were caused by root uptake of soil nutrients with the help of microbial

activity, and/or influenced by root exudates (Hinsinger, 2005; Huang *et al*., 2014). Plants may release components of root exudates of several low and high molecular weight organic compounds such as sugars, organic acids, amino acids, and phenolics into the rhizosphere (Hinsinger, 2005; Marschner and Romheld, 1996). These compounds released by the root can lead to dissolution of primary minerals and precipitation or crystallization of secondary compounds and/or minerals and eventually transformation of mineral components in the rhizosphere (Cabala, 2004). Plant Growth Promoting microbes also increased concentration in the rhizosphere soil and deliberate the plants with beneficial effects such as uptake of nutrients by siderophore, solubilization of inorganic phosphate, fixation of nitrogen, and extracellular enzymes producing ability to suppress plant pathogens (Gupta *et al*., 2000; Weller *et al*., 2002; Kloepper *et al*., 2004; Yang *et al*., 2009; Mendes *et al*., 2011; Tahir *et al*., 2016). Bambara and Ndakidemi, 2010 reported a significant increase in soil pH, Ca, and Na following Rhizobium inoculation in *Phaseolus vulgaris*.

Selected isolate AB832 on the bean and maize crop plant under greenhouse for 20 days showed significant changes in rhizospheric soil parameters of nitrogen (N), phosphorus (P), potassium (K), soil organic carbon (SOC) and pH. The highest rhizosphere K level was recorded in bean inoculated with isolate AB832 compared to the uninoculated, control soil while, the lowest level of N, P, SOC and pH with isolate AB832 was recorded. Our results predicted that isolate AB832 may be optimally utilize the available nutrients of the soil in the initial stage to be used or released in lateral stage of the plant growth. Therefore, this could be a result of utilization of the available N, P, SOC and changes in pH, the development of root systems of bean was showed decrease in root length, root surface area, fresh weight and dry weight. The selected actinomycete isolate in present study were capable of utilizing soil nutrients by producing phytohormone, phosphate solubilizing, nitrogen fixation, production of ammonia and enzymes and in turn exhibit PGP activities that were extremely beneficial for plants. Moreover, soil bacteria are known to be attracted to root exudates and mucilage present in living plant roots and in soil (Lugtenberg and Kamilova, 2009).

In the present study, the rhizosphere soil pH, N, K, SOC was significantly higher in isolate AB832 inoculated maize over uninoculated one throughout the maize plant growth. This result indicating isolate AB832 ability to influenced rhizospheric soil properties of maize. The enhancement of PGP properties in the rhizospheric soil have played important role in response of plant growth and development. Microbial production activity produces favourable conditions to maize plant. Therefore, *Streptomyces* spp. (isolate AB832) treated on the crop rhizosphere were able to survive and confer PGP. There was evidences that treated with *Streptomyces* sp. increased the N, P, SOC and enzymes of soil (Sreevidya *et al*., 2016).

Hence, this may imply that this AB832 strain had competitive advantage and positively affected the growth of inoculated plants and influenced the soil properties of the plant rhizosphere can be used as a potential biofertilizer under the field conditions.

### 5.6. Rhizospheric soil properties analysis of the study area

In the present study, there was a variation in rhizosphere soil nutrient element among the two different *jhum* cultivation sites. Significantly higher of the chemical properties was observed in rhizospheric soil of Reiek *jhum* cultivation compared with the rhizosphere soil collected from Tanhril *jhum* cultivation (Fig.4.18). Rhizosphere soil chemical properties that were significantly increased in K, P, SOC in Reiek *jhum*. Whereas, rhizosphere soil of Tanhril *jhum* cultivation significantly increase N level in the rhizosphere soil (Fig.4.18).

#### 5.6.1. Nitrogen (N)

In the present study, nitrogen content was higher in Tanhril *jhum* rhizospheric soil compared to the Reiek *jhum* rhizospheric soil (Fig.4.18). Maximum nitrogen content in the Tanhril *jhum* may be due to the production of $N_2$ fixation of other bacteria while the N content in the Reiek *jhum* may be due to the *Streptomyces* sp. for example, in our study, strain AB832 isolated from Reiek *jhum* rhizospheric soil was showed ability to fix atmospheric $N_2$. Nitrogen fixation is the ultimate cause behind increased nitrogen concentration in rhizosphere (Saharan and Nehra, 2011). It was

also reported that increase in rhizosphere soil N content with application of PGPR by Cakmakci *et al*. (2007).

### 5.6.2. Phosphorus (P)

P is second important soil nutrient element for plant growth and development. Present result showed higher levels of P in the rhizosphere soil of Reiek *jhum* than Tanhril soil (Fig. 4.18). There may be several possible factors for increased concentration of nutrients in the rhizosphere soils of Reiek *jhum*. Firstly, it may be due to less human population in the Reiek *jhum* cultivation areas and easy availability of natural forest for *jhum* cultivation which favoured the availability of most soil nutrients. Increased availability of nutrients in the soil provides establishment of rhizosphere that may develop higher mineral nutrients in the rhizospheric soil and eventually increased yield. Soil nutrient heterogeneity of shifting cultivation is influenced by the presence of vegetation in a habitat and depends upon tree species (Ibrahim *et al*., 2016). Change in vegetation composition is one of the factors for changes in the status and release of nutrients in soil and chemical composition of soil (Rhoades, 1997). Thus, the undisturbed natural forest sites produce more litter by adding plant nutrients into the soil provides stable nutrient cycling and enrich soil fertility (Tokyo and Ramakrishnan, 1983). Slashing and burning is practiced to prepare the land for cultivation to integrate nutrients into the soil that have been accumulated in vegetation (Nath *et al*., 2016). Thus shifting cultivation practice in less human population density and lower disturbed areas have more stable soil fertility and generate sustainable shifting cultivation. Secondly, mineralization activities of phosphate solubilizing rhizospheric microorganisms make nutrients available in the soil. Phosphorus is unavailable to the plant directly due to it is usually deficient in the soil because it is fixed in soil layers. (Wang *et al*., 2009; Shenoy and Kalagudi, 2005; Khan *et al*., 2009; 2014). This insoluble phosphorus can be fixed with calcium ($Ca_3PO_4)_2$, aluminum ($Al_3PO_4$) and iron ($Fe_3PO_4$) and turned to soluble forms by P-solubilizing organisms (Gupta *et al*., 2007; Song *et al*., 2008; Sharma *et al*., 2013). P-solubilizing soil microbes have the ability to mineralize complex compounds (Bishop *et al*., 1994; Toro, 2007). P-solubilizing microorganism's release of different organic acids by can lead to

acidification of microenvironments (Maliha *et al*., 2004) and consequently replacement of P ions with cations (Goldstein, 1994; Mullen, 2005; Trivedi and Sa, 2008). Phosphorus solubilizing microorganism has gained importance due to their multifunctional capabilities enhancing phosphorus availability for the plants. Some P-solubilizing microbes are also responsible for production of siderophore (Tank and Saraf, 2003), indole acetic acid and gibberellin (Souchie *et al*., 2007), antibiotics (Taurian *et al*., 2010).

### 5.6.3. Potassium (K)

Potassium is the third important plant nutrient. It plays a key role in the growth production of plants. For the development of root system adequate supply of K is required, poor K in the plants cause poorly developed roots and slow growth, ultimately produce lower yields and small seed production (McAfee, 2008, White and Karley, 2010) and the increased susceptibility to diseases (Amtmann *et al*., 2008, Armengaud *et al*., 2010) and pest (Amtmann *et al*., 2006, Troufflard *et al*., 2010). Rhizospheric bacteria activity involves in soil processes such as exudation of soluble compounds, storage and release of nutrients, mobilization and mineralization of nutrients, soil organic matter decomposition and solubilization of K (Rajawat *et al*., 2012, Parmar and Sindhu, 2013, Archana *et al*., 2013, Zeng *et al*., 2012, Verma *et al*., 2012a, Verma *et al*., 2012b, Abhilash *et al*., 2013), and phosphate solubilization, nitrogen fixation, nitrification, denitrification, and sulfur reduction (Khan *et al*., 2007, Diep and Hieu, 2013). Bacteria ability to solubilized potassium (KSB) and they can convert the insoluble or mineral structural potassium compounds into soluble forms in soil as a soil solution and make them available to the plants (Zeng *et al*., 2012). K-solubilizing bacteria are potential to release K from insoluble minerals (Sugumaran and Janarthanam, 2007, Basak and Biswas, 2009, Basak and Biswas, 2012, Kalaiselvi and Anthoniraj, 2009, Parmar and Sindhu, 2013, Zarjani *et al*., 2013, Prajapati *et al*., 2013, Zhang *et al*., 2013, Gundala *et al*., 2013, Archana *et al*., 2012, Archana *et al*., 2013, Sindhu *et al*., 2012). Many researchers have found that K-solubilizing bacteria were able to improve soil nutrients and structure, beneficial for plant growth through suppressing pathogens. The diverse K-solubilizing microbes are present in rhizospheric soils which promote the plant growth (Sperberg,

1958). K-solubilizing bacteria synthesized various organic acids results in acidification of the microbial cell and its surroundings environment which promote the solubilization of mineral K.

In the present study, Reiek *jhum* rhizospheric soil showed an increase in K in the rhizosphere soil compared to Tanhril *jhum* rhizopsheric soil (Fig.4.18). This increase may have significant effects on K caused by the PGP potential actinomycetes of the soil rhizosphere.

### 5.6.4. Soil organic carbon (SOC)

SOC content was higher in rhizospheric soil of Reiek *jhum* than Tanhril *jhum* site (Fig.4.18). The variations in the rhizosphere SOC content may be related to changes in plant species, extent of root exudation, microbial growth and SOC decomposition. Previous studies reported that the amount of root exudates released was positively correlated with microbial growth and SOC decomposition (Dijkstra and Cheng, 2007a; Phillips *et al*., 2011; Bengtson *et al*., 2012). Rhizodeposits consist of organic C such as sugars, organic acids, mucilage, sloughed cell walls and root hairs, but can also include nitrogen N-containing organic compounds such as amino acids (Hutsch *et al*., 2002). This study provides further evidence on the role of *Streptomyces* sp. isolated from Reiek *jhum* on influencing the SOC in the rhizosphere. Further, PGPR microbial inoculants have reported to increase SOC in the rhizosphere of *A. sativa*, *M. sativa*, and *C. sativus*. (Li *et al*., 2020). Our results showed changes in pH in Reiek and Tanhril *jhum* rhizospheric soil (Fig.4.18). The plant species variation could be attributed to the effect on rhizosphere pH. McLay *et al*., 1997; Tang *et al*., 1999 demonstrated that the greater capacity of chickpea to acidify the rhizosphere could be explained by its apparent excess uptake of cations over anions during $N_2$ fixation. According to Rousk *et al*., 2010 study, microbes' acidobacter were reported to be highly associated with soil pH. It has been investigated that soil pH has a noticeable influence on the composition of the microbial community. Previous study by Wu *et al*., 2019 found that the plant–microbe interactions contribute to increase acidity and create a new environment to mediate changes in the microbial community structure in the *R. pseudostellariae* rhizosphere under continuous monoculture regimes.

## 5.7. Identification of PGP potential actinomycetes strains by 16S rRNA gene sequencing and phylogenec analysis

All the 11 rhizopsheric actinomycetes of PGP positive were characterized by PCR amplification of 16S rRNA gene. The DNA sequence of most isolates showed 99-100% identity with BlastN sequences and phylogenetic analysis based on 16S rRNA gene amplification showed that *Streptomyces* formed a major group consistent with previous studies (Passari *et al*., 2015a; Zhao *et al*., 2011). Actinomycetes strains like *Streptomyces* spp. and *Micromonospora* spp. were reported as best to colonize the plant rhizosphere showing PGP potentiality (Franco-Correa *et al*., 2010). The composition of rhizospheric actinomycetes particularly *Streptomyces* as revealed by phylogenetic trees was more diverse as similar to rhizopsheric actinomycetes isolated from wheat and tomato (Anwar *et al*., 2016). All the *Streptomyces* isolates fall under one major clade with an exception of *Micromonospora* sp. (Zhao *et al*., 2011). One strain of actinomycetes which was identified under genus *Streptomyces* sp. (AB832, accession number MN326863) induced phosphate solubilization ability along with its capacity to produce IAA, ammonia, amylase, cellulose and nitrogen fixation. Other potential actinomycetes identified were: *Streptomyces venezuelae* (AB757, accession number MN326854), *Micromonospora auratinigra* (AB726, accession number MN326855), *Streptomyces avellaneus* (Ab761, accession number MN326856), *Streptomyces seoulensis* (AB770, accession number MN326857), *Streptomyces scabiei* (AB774, accession number MN326858), *Streptomyces vinaceus* (AB782, accession number MN326859), *Streptomyces venezuelae* (AB759, accession number MN326860), *Streptomyces mirabilis* (AB828, accession number MN326861), *Streptomyces scabiei* (AB784, accession number MN326862), *Streptomyces* sp. (AB788, accession number MN326864).

A fragment of the 16S-rRNA gene of AB832, which exhibited significant PGP activity, was sequenced and the sequences were deposited in NCBI GenBank (Accession number MN326854 -MN326864). Isolate AB832 were selected as the best PGP potential among the total isolates for plant growth promotion assay. Based on the obtained sequence, AB832 was identified as *Streptomyces* sp. The 16S

sequence of AB832 exhibited 99% similarity to *Streptomyces* sp. (Table 4.3, fig.4.19).

Shifting cultivation (*jhum*) has adopted traditionally in Northeast India. The *jhummias* are known to produce more than 30 indigenous crops along with other species. These major crops under *jhum* cultivation may be adapted in the soil of this region because of their PGP potential, and therefore, *jhummias* selected these major crops in the practice of *jhum* cultivation. PGP potential rhizospheric actinomycetes may be one of the responsible factors for traditional crops to become adapted under shifting cultivation by enhancing the soil fertility, plant growth and crop yield. Therefore, microorganism's actinomyces native to *jhum* cultivation were isolated and screened for PGP properties. Isolates that exhibited production of PGP properties and showed the effect of PGP in bean and maize crops under *jhum* soil of this region have been tested. The inoculation of these indigenous microbes can easily survive in the crop plants of the region and may be useful in providing advantage to the management of agricultural crops that are grown in the *jhum* fields. The 16S rRNA gene is an advance tool for evaluating bacterial phylogeny which has rapidly changed bacterial taxonomy (Olsen and Woese, 1993). 16S rRNA gene sequence technique has provided data that have been advanced for use in the field of microbial ecology to evaluate the members of diverse microbial communities (Giovannoni *et al*., 1990; Hugenholz *et al*., 1998). Isolation and identification of PGP active actinomycetes at molecular level is crucial for understanding the indigenous potential actinomycetes. Molecular results suggest higher levels of species diversity of actinomycetes (i.e. *Streptomyces* and *Micromonospora*) in the present study. This study strengthens the microbial taxonomy in a broad sense of microbial classification. In the future, efforts will be made further explore these isolates for the development of consortium as bioinoculants for sustainable *jhum* cultivation. Also, it is necessary to understand the plan-microbe interaction and their role in the major crop plants of *jhum* cultivation in relation to the soil. This study suggests that rhizospheric soil fertility and PGP potential rhizopsheric microbes may profoundly affect the growth of major crops under *jhum* cultivation in Mizoram, northeast India.

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# Chapter 6

## Conclusion

Present agricultural practice using synthetic chemicals for enhancing crop yield have largely affecting the soil fertility and production capacity of the ecosystem. Therefore, microbial substitute in the form of biofertlizer based on natural sources for the sustainable plant growth in agriculture is required. In this respect, rhizospheric actinomycetes have significant potential to be explored because of their properties beneficial for enhancing the plant growth. The result of the present study revealed that the rhizosphere soil of crops under shifting cultivation have different types of actinomycetes with *Streptomyces* spp. as the most abundant and common. *In-vitro* screening of the isolates demonstrated that actinomycetes colonizing major crop plants promote plant growth through production of plant growth regulators (IAA, siderophore, ammonia, amylase, catalase), phosphate solubilization, fixing nitrogen.

*In-vivo* screening of *Streptomyces* sp. AB832 isolate demonstrated a significant PGP activity in local crops (i.e. bean and maize) under greenhouse experiment. The PGP activity enhanced shoot surface area, shoot average length of bean plant with treatment of *Streptomyces* sp. AB832 isolate compared to control. Changes in shoot average diameter did not vary significantly. There was an increase in shoot fresh weight with treatment isolate AB832 as compared to control. Interestingly, root surface area and average length were also recorded lower in treatment with isolate AB832 than control that may be due to their complex mechanisms of interaction within short period of time. Root average diameter were recorded maximum with treatment isolate AB832 than control. *Streptomyces* sp. AB832 isolate displayed increase shoot surface area and shoot average diameter of the maize when compared with the control after 20 days of sprouting. Shoot fresh weight and dry weight of maize were recorded maximum in treatment than control. Significantly enhanced higher growth in root surface area, root average diameter and root average length of

maize with treatment when compared to control. This study found increased root dry weight in treatment pots compared to control.

This result suggest that rhizospheric actinomycetes colonized in major crop plants can increase shoot portion and root portion in bean and maize plants and such an increase may confer advantages to the host plant with respect to health and overall growth. These *streptomyces* spp. have predominant activity on plant growth promotion with respect to indigenous crop plant. All the tested *Streptomyces* strains not only colonized on the roots of the crop plants but also proliferated and enhanced PGP in bean and maize of the local crop plants. Association of actinomycetes in the crop plant rhizosphere deliberates several benefits to plants like production of ammonia, siderophores and phosphate solubilization, extracellular enzymes (amylase and catalase), phytohormones (IAA), nitrogen fixation. The isolation of microorganisms actinomycete from shifting cultivation sites offer microorganisms with unusual properties and activities. Many soil microbial communities might have been eliminated due to fire event and only few organisms could resist the environmental stresses as result of their unique functionality which arises out of their biological system that produces potential PGP to make them adapt to such environments.

Rhizospheric soil variables in the present study showed significant variation with respect to study sites and crop plants. There were significant interactions between actinomycetes and rhizosphere soil of major crops of shifting cultivation. Rhizospheric actinomycetes inoculation altered most of the chemical properties of the rhizosphere soil of bean and maize in this study. The rhizosphere soil chemical properties such as pH, nitrogen (N) and potassium (K) were decreased with the actinomycetes treatment in bean over the control. This result indicates the requirement of particular soil nutrients (N and K) by actinomycete in their early stage of interaction with bean crop for their next stages of plant growth. Phosphorus did not change with actinomycete treatment in the early stage of plant growth. However, increase in soil organic carbon with actinomycete treatment occurred in comparison to control. Rhizosphere soil chemical properties of maize showed significant increase

in the pH, nitrogen, potassium, soil organic carbon in actinomycete treated pots compared to control.

The use of molecular technique adds more precision and accuracy to the phylogenetic identification and also to the true reflection of microbial diversity. All the PGP potential rhizospheric actinomycetes isolates were characterized by PCR amplification using the 16S rRNA gene. The DNA sequence of the isolates showed 97–100% identity with BlastN sequences and phylogenetic analysis based on 16S rRNA and the gene amplification revealed that *Streptomyces* formed a major group followed by *Micromonospora*. Molecular technique using 16S rRNA PCR amplification and sequencing provided a reliable tool for the detection of similarities and differences in the relationships among different isolates in the same bacterial genus and species. 16S rRNA sequencing and phylogenetic construction have proved a very useful tool to classify highly related strains and has been applied to study the genetic diversity at the species level among the rhizospheric actinomycetes isolates.

The plant microbe interaction in the rhizosphere provides unique biological position that occupies the abundance and diversity of microbes that are influenced by the exudates of plant roots. Present study revealed that rhizospheric actinomycetes from major crops under shifting cultivation provided a diversity of actinomycetes which belonged to genus *Streptomyces* spp. having potential for producing PGP properties and changing the soil environment. Thus, exudation of carbon from these major crop plants in the present study may be the important factor on development of soil actinomycetes communities with potential of PGP properties that influence rhizosphere soil characteristics. Indigenous major crop plants are likely to easily adapt to the habitat because their plant root exudation process that favours the indigenous microorganisms and soil characteristics to enhance soil fertility for sustainable traditional agricultural practices. This result showed that major crop plants species under shifting cultivation of Mizoram associated with actinomycetes with peculiar properties and may have the capacity to exude specific compounds which significantly influence the rhizosphere of plant species in the soil.

Potential PGP associated root actinomycetes (i.e. *Streptomyces* and *Micromonospora*) can be employed to increase soil fertility and crop productivity in

shifting agriculture. Efficiency of these actinomycetes may be improved with further studies so that a suitable bio-inoculant can be developed for the crops under shifting cultivation. Target actinomycete strains in the present study have the potential to improve plant growth and soil health. Thus, inoculation of these rhizospheric actinomycetes species to the locally available crop plants under may positively affect plant growth under the shifting cultivation in Mizoram. Further studies would be required to develop multiple combinations for better and more suitable bioformulations with robust *in-vivo* trials for the large-scale transformation of the result.

# Bio-data


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## ACADEMIC QUALIFICATION

| Qualification | Passing Year | University/Institution | Percentage |
|---|---|---|---|
| **Master of Philosophy (M.Phil)** | **2014-2016** | **SCHOOL OF LIFE SCIENCE, MIZORAM UNIVERSITY, MIZORAM** | **75%** |
| **M.Sc FORESTRY** | **2011-2013** | **SCHOOL OF EARTH SCIENCE, MIZORAM UNIVERSITY, MIZORAM** | **72.33%** |
| **B.Sc (Chemistry, Botany, Zoology, EVS)** | **2008-2011** | **DON BOSCO COLLEGE, TURA** | **44%** |
| **HSLC (XII)** | **2005-2007** | **DON BOSCO COLLEGE, TURA** | **48%** |
| **SSLC ( X )** | **2004-2005** | **ST. MARY'S HIGHER SECONDARY SCHOOL, TURA** | **58%** |

## EXPERIENCE DETAILS

- Worked as project fellow at "Soil-Plant-Beetle interactions in disturbed and undisturbed areas of Mizoram, Northeast Himalayan, India" at the DBT-State Biotech Hub's, Department of Biotechnology, Mizoram University, Aizawl from 01/09/2013 to 31/05/2014.

## PUBLICATIONS AND OTHER ACHIEVEMENTS

- Momin, M. D. and Tripathi, S. K. (2018). Rhizospheric actinomycetes from major crop plants under shifting cultivation of Mizoram, Northeast India. De Dulal, S. Roy, G. C. Bera (eds.), Biotechnology and Nature, Kabitika, 260pp. ISBN 978-93-87602-66-3.

- Ghosh, S., Momin, M. D. and Tripathi, S. K. (2018). Rhizospheric actinomycetes from major crop plants under shifting cultivation of Mizoram, Northeast India. De Dulal, S. Roy, G. C. Bera (eds.), Biotechnology and Nature, Kabitika, 260pp. ISBN 978-93-87602-66-3.

- Momin, M. D. and Tripathi, S. K. (2019). Actinomycetes from Shifting Cultivation (Jhum) of Mizoram, Northeast India. *Environment and Ecology*, 37(3B): 1081-1085.

- Momin, M. D. (2019). Role of Actinomycetes in agriculture. Agriculture and Food:e-Newsletter, 1(5). Article id: 11158. ISSN: 2581-8317.

- Momin, M. D. and Tripathi, S. K. (2020). Rhizosphere *Streptomyces* Species from Major Crop Plants of Shifting Cultivation, Northeast India. *Indian Journal of Ecology*, 47(2): 570-574.
- Momin, M.D and Ibrahim, K.S. (2020). Endophytic Bacteria Isolated from Ageratum conyzoides L. and Mikania micrantha Kunth of Mizoram, Northeast India. *National Academy Science Letters*

## CONFERENCE, TRANING AND WORKSHOPS ATTENDED

- Poster presented on "Study of rhizospheric actinomycetes from shifting cultivation of Mizoram, Northeast India" in the 12$^{th}$ Annual) Convention of Association of Biotechnology and Pharmacy (ABAP) and International Conference on Biodiversity, Environment and Human Health: Innovations and Emerging Trends (BEHIET 2018) organized at the School of Life Sciences, Mizoram University, Aizawl, Mizoram 796004 during November 12 to 14, 2018.

- Poster presented on "Identification of Rhizospheric Actinomycetes Isolated from Crop Rice" in the International Conference on Chemistry and Environmental Sustainability (ICCES-2019) on 19$^{th}$-22$^{nd}$ February 2019, Department of Chemistry, Mizoram University.

- Oral Presented on "Plant Growth Promoting Potential of Rhizosphere Soil Actinomycetes Isolated from Major Crops Under Shifting Cultivation of Mizoram, Northeast India" in the National Seminar on Recent Trends in Ecological Research (RTER-2019) 5$^{th}$ -7$^{th}$ March, Department of Ecology and Environmental Science and Centre for Biodiversity and Natural Resource Conservation Assam University, Silchar.

- Awarded best oral presentation on "Plant Growth Promoting Potential of Rhizosphere Soil Actinomycetes Isolated from Major Crops Under Shifting Cultivation of Mizoram, Northeast India" in the National Seminar on Recent Trends in Ecological Research (RTER-2019) 5$^{th}$ -7$^{th}$ March, Department of Ecology and Environmental Science and Centre for Biodiversity and Natural Resource Conservation Assam University, Silchar.

- Poster presented on "Rhizospheric actinomycetes isolated from major crop plants under shifting cultivation of Mizoram, Northeast India" in the international workshop on Novel Methods for Nutrient Management in Shifting

Cultivation in NE India: Balancing the old and new (22nd-24th January, 2020), Mizoram University, Aizawl.

- Best article award on "Role of Actinomycetes in agriculture" article no. 11158 in volume1 Issue 5 in Agriculture and Food: e-Newsletter, 2019.

- Attended a national workshop on **"Hands on Training on DNA barcoding and phylogenetics"** held on 20-25th March 2017 organized by Advanced level State Biotech-Hub Facility, Department of Biotechnology, Mizoram.

- Attended a worshop on **"Training and Awareness programme on protection of plant varieties and farmers Rights"** held on 28-29th March 2017 organized by Department of Forestry and Department of Horticulture, Aromatic and Medicinal Plants, Sponsored by PPVFRA, Ministry of Agriculture and Farmers Welfare, Govt. of India and Mizoram University, Aizawl.

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| | |
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## Declaration:

I hereby declare that all the information mentioned above is true to the best of my knowledge and belief. I will be solely responsible if any of the information is found wrong.

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DEPARTMENT : Forestry

TITLE OF THESIS : Characterization of rhizospheric actinomycetes from major crop plants and their plant growth promoting properties under jhum fields of Mizoram

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1. Date of Approval in DRC: 10th April 2017

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EXTENTION (IF ANY) : NO

(HEAD)

Department of Forestry

# ABSTRACT

# CHARACTERIZATION OF RHIZOSPHERIC ACTINOMYCETES OF MAJOR CROP PLANTS AND THEIR PLANT GROWTH PROMOTING PROPERTIES UNDER JHUM FIELDS OF MIZORAM

**A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY**

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**2020**

**Abstract**

Rhizospheric actinomycetes (i.e. *Streptomyces* and *Micromonospora*) were found to be the most promising plant growth promoting (PGP) strains. The main objective of the present study was to isolate rhizospheric actinomycetes from major crop plants under shifting cultivation (*jhum*). This study was carried out in two shifting cultivation areas, Reiek, Mamit district and Tanhril, Aizawl district of Mizoram, Northeast India. Shifting cultivation is dominant land use practice in Mizoram. Rhizosphere soil was collected from major crops viz., rice, maize, yam and beans of the study site. Isolation of actinomycetes were followed by standard method of serial dilution from $10^1$-$10^7$ and inoculated on IM8, International Streptomyces Project 2 (ISP2), Starch Casein Agar (SCA) and Cross-streak media (CSM) medium. Inoculated plates were incubated at 28˚C for 1-4 weeks. The isolates were characterized morphologically according to the Bergey's Manual of Determinative Bacteriology and were subjected to *in-vitro* screening for various plant-growth promoting (PGPRs) traits like phosphate solubilization, ammonia production, indole-3-acetic acid (IAA), siderophore production, and nitrogen fixation, amylase and catalase production. Dry, fuzzy, filamentous, entire, irregular, convex or raised, sticky-hard, colony with 0.3mm to 1.5mm in dia., size, pigmentations and slow growth formation of actinomycetes were observed. Total 35 strains of rhizospheric actinomycetes were isolated in which 3 (8.5%) actinomycetes were obtained from *jhum* cultivation of Tanhril and 32 (91.4%) actinomycetes were isolated from *jhum* cultivation of Reiek. Based on media used, 12 isolates (34.2%) form CSM followed by 11 isolates (31.4%) in SCA media, 8 isolates (22.8%) were obtained from ISP2 media and 4 isolates (11.4%) from IM8. Isolated rhizospheric actinomycetes strains were identified as majority belonged to the genus *Streptomyces* sp. followed by few were belonged to *Micromonospora* sp. Eleven isolates which showed PGP activity at least one trait of the tested PGP properties. Among the 35 isolates, 11 strains were able to formed clear zones on modified Pikovskaya agar plates, clear zone formation were indicative of phosphate solubilization. Out of 35, only 2 strains were able to produce ammonia. Out of total, 4 strains were showed the blue colour of the medium to orange or presence of yellow to light orange halo surrounding the colony indicates

the production of siderophore. Out of 35, 5 isolates was observed as the development of a pink to red colour indicates positive for IAA production. Out of total isolates, only 1 strain were able to grow on free- nitrogen medium and showed turning from yellow to green color were confirmed to have the capacity of fixing atmospheric nitrogen. Out of total isolates, 8 isolates were observed bubbles productions drop of 3% $H_2O_2$ was added in the culture tubes. Among 35 isolates, only 2 strains were amylase producers by forming clear zone around the colony of starch agar. Among the 11 positive PGP isolates, the most active PGP producer strain were selected for *in-vivo* screening, seed germination, and plant growth-promoting. *In-vivo* experiments were conducted to determine the physiological responses of maize (*Zea mays* L.) and bean (*Phaseolus mungo* L.) seed germination and seedlings by inoculation with most potent rhizospheric actinobacterium e.g. *Streptomyces* sp. isolate AB832 under greenhouse conditions. There was a significant effect of the AB832 isolate on the germination rate of bean and maize seeds compared to non-inoculated seeds. With treatment of AB832 isolate, 75% of bean seeds germinated and in control the number of seeds germinated was 50%. In maize plant, 62.5% seeds were able to germinate and 37.5% seeds were germinated in control. In bean, shoot portion of bean particularly shoot surface area, shoot average length, increases in shoot fresh weight were recorded significantly higher than the control. Whereas, the root portion like root surface area and average length, higher root fresh weight and dry weight decreased with treatment AB832 isolate when compared to control. Consequently, root average diameter was recorded with treatment isolate AB832 compared to control. Data was statically analysed using a one way ANOVA and least significant difference (LSD) tests at $p \leq 0.05$ ($LSD_{0.05}$ = 2515.1). Highly correlation between treatment and control of shoot (0.99869953=1), root (0.97021169=1). In maize, treatment with AB832 isolate exhibited increase shoot surface area and average diameter, shoot fresh weight and dry weight. Significantly enhancement occurred in root surface area, root average diameter and root average length with treatment AB832 isolate compared to control. Data were statically analysed using a one way ANOVA and least significant difference (LSD) tests at $p \leq 0.05$ (LSD 0.05 = 1618.2). Highly significant positive correlation between treatment and control in shoot (0.976179=1) and root (0.99857=1). The rhizospheric soil analysis of bean

were resulted higher amount of soil organic carbon and there was no significant difference in phosphorus in case of bean rhizospheric soil and small increase occurred in the amount of available nitrogen, potassium, and pH in the treatment AB832 isolate. The rhizopsheric soil analysis of maize exhibited increase in the pH, nitrogen, potassium, soil organic carbon in treatment compared to control. There was no significant difference in available phosphorus in treatment and control pots. Data was statically analysed using a one-way ANOVA and least significant difference (LSD) tests at $p \leq 0.05$ ($LSD_{0.05} = 546.8$). Highly correlation between treatment bean and control bean (0.985195738=1), between treatment maize and control maize (0.975959392=1). Rhizospheric soil analysis from two study areas were revealed that phosphorus and potassium was significantly higher in *jhum* cultivation of Reiek compared to *jhum* cultivation of Tanhril and nitrogen was lower in *jhum* cultivation of Reiek as compared to *jhum* cultivation of Tanhril. Data was statically analysed using a one-way ANOVA and least significant difference (LSD) tests at $p \leq 0.05$ (LSD0.05 = 205) for rhizospheric soils of Reiek and Tanhril *jhum* soil. PGP positive 11 isolates were selected for 16S rRNA sequencing and phylogenetic analysis. Isolates were differentiated into 2 different families and 2 different genera. Majority of the isolates belonged to family Streptomycetaceae which comprised 90% of the total isolates, followed by family Micromonosporaceae (9%). Based on the similarity percentage (99-100%) of 16S rRNA gene sequences of the isolated organisms, 10 isolates belong to *Streptomyces* and 1 isolate belonged to *Micromonospora* genera. The present study offered significant potential for microbial consortia from the combination of these strains that can be useful as bioinoculants for sustainable shifting agricultural in Northeast India.

**Keywords:** Rhizosphere actinomycetes; shifting cultivation; major crops; PGP properties; 16S rRNA sequencing; phylogenetic analysis