Studies of endophytic actinomycetes associated with medicinal plants for their plant growth promoting activities

Thesis submitted in partial fulfillment of the requirement of the Degree of Master of Philosophy in Biotechnology

By

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M. Phil. Registration No: MZU/M. Phil/200 of 22.05.2015

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DECLARATION OF THE CANDIDATE

I, Marcy D. Momin, hereby declare that the subject matter of this dissertation entitled " Studies of endophytic actinomycetes associated with medicinal plants for their plant growth promoting activities" is the record of work done by me, that the contents of this dissertation 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 dissertation 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 Master of Philosophy in Biotechnology.

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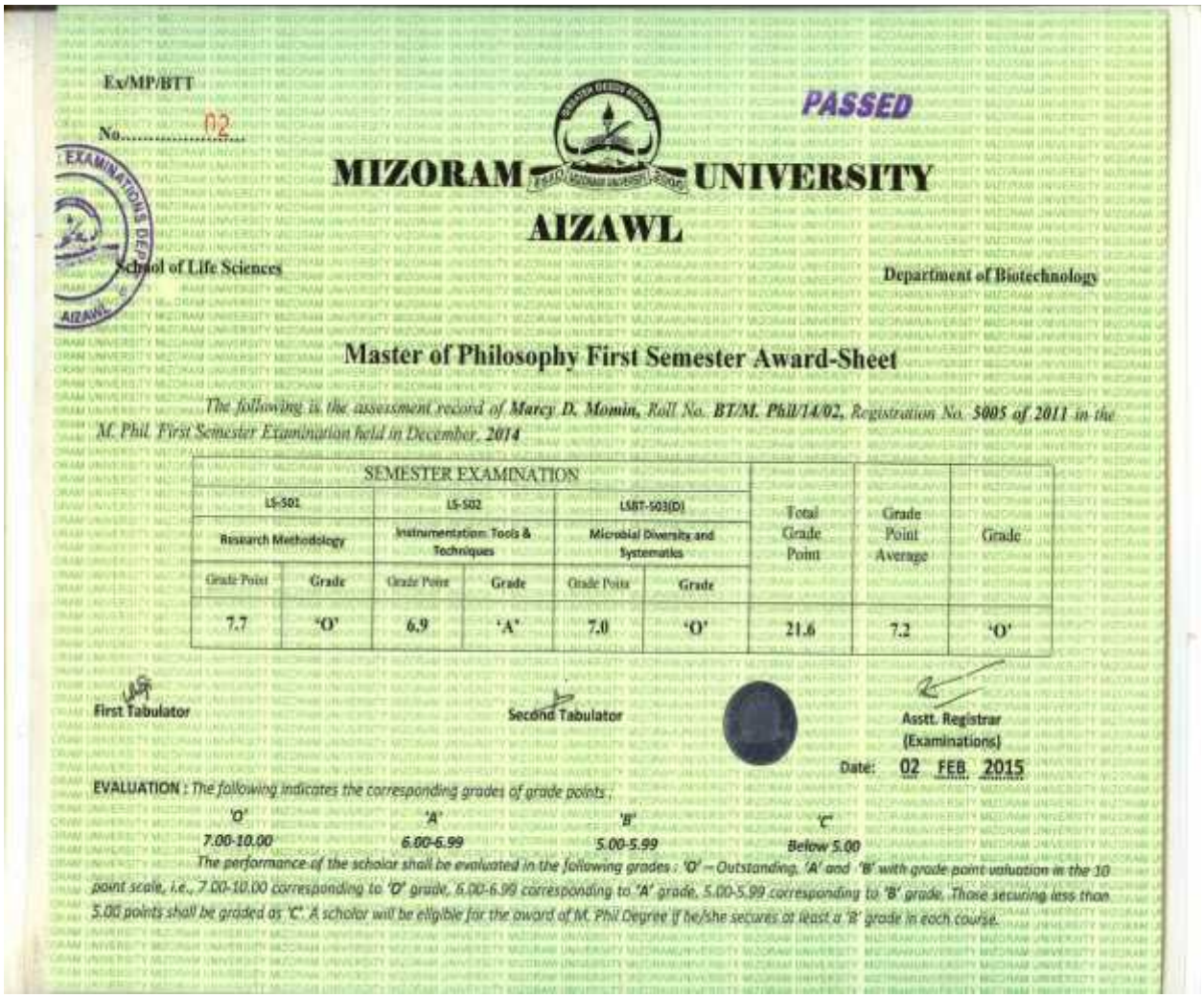

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SEMESTER EXAMINATION

LS-501		LS-502		LSBT-503(D)		Total Grade Point	Grade Point Average	Grade
Research Methodology		Instrumentation: Tools & Techniques		Microbial Diversity and Systematics				
Grade Point	Grade	Grade Point	Grade	Grade Point	Grade			
7.7	'O'	6.9	'A'	7.0	'O'	21.6	7.2	'O'

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EVALUATION : *The following indicates the corresponding grades of grade points :*

'O'	'A'	'B'	'C'
7.00-10.00	6.00-6.99	5.00-5.99	Below 5.00

The performance of the scholar shall be evaluated in the following grades : 'O' – Outstanding, 'A' and 'B' with grade point valuation in the 10 point scale, i.e., 7.00-10.00 corresponding to 'O' grade, 6.00-6.99 corresponding to 'A' grade, 5.00-5.99 corresponding to 'B' grade. Those securing less than 5.00 points shall be graded as 'C'. A scholar will be eligible for the award of M. Phil Degree if he/she secures at least a 'B' grade in each course.

Dedicated to

my parents and my family

ACKNOWLEDGEMENT

First and foremost, I thank the Almighty God Jesus for His blessing and guidance in my life.
I owe my deepest thanks and heartfelt gratitude to Dr. Bhim Pratap Singh, Supervisor, Department of Biotechnology, Mizoram University, Aizawl, Mizoram, for his excellent guidance, assistance and sharing his precious treasure of knowledge throughout my M. phil research.

I express my heartiest gratefulness to The Head, Dept of Biotechnology, Mizoram University and other faculty members who have shown great co-operation and helping all necessary things during my research.

I am very much indebted to Mr. Ajit Kumar Passari, Ph.d research scholar, Department of Biotechnology, Mizoram University, who have shown great co-operation in my experimental work. I also extend my heartfelt thanks to my laboratory mates, who spared their valuable time and gave me constant support and necessary help.

I am thankful to the Department of Biotechnology, New Delhi again, for establishment of DBT-BIF centre and DBT-state Biotech Hub in the Department, which also has been used for the present study.

At last I express my heartiest gratefulness to my parents and family for their encouragement and support financially and their sincere prayers.

I am equally thankful to the various funding agencies like Department of Biotechnology, New Delhi, University Grants Commission, New Delhi and Department of Science and Technology, New Delhi for funding.

ABBREVIATIONS

U, ml	: Unit, milliliter
µM	: Micromole
REP-PCR	: Repititive enterobacterial Polymeric Chain Reaction
ERIC-PCR	: Enterobacterial repetitive intergenic consensus PCR
PCR	: Polymeric Chain Reaction
FEG-SEM	: Field-Emission Gun-Scanning Electron Microscopy
IAA	: Indole acetic acid
DNA	: Deoxyribonucleic acid
DNS	: Dinitro Salicyclic Acid
UPGMA	: Unweight Pair Group Method using Arithmetic Average
NTSYS	: Numerical Taxonomy System
NCBI	: National Centre for Biotechnology Information
ISP5	: International Streptomyces Project 5
ISP1	: International Streptomyces Project 1
AIA	: Actinomycetes Isolation Agar
SCA	: Starch Casein Agar
SCNA	: Starch Casein Nitrate Agar
TWYE	: Tap Water Yeast Extract Agar

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CHAPTER 1

INTRODUCTION

Actinomycetes are characterized as aerobic,gram-positive bacteria that form branching filaments or hyphae and asexual spores with high G+C (guanine+cytosine) content in there genome and are ubiquitous in nature. They are widely distributed in a variety of natural and manmade environments, particularly constituting a significant component of the microbial population in temperate forest soils (Debananda *et al*., 2009; Lam, 2006; Ndonde and Semu, 2000; Watve *et al*., 2001).Fewof them have an aerial mycelium that extends above the substratum and forms asexual, thin-walled spores called conidia or conidiospores at the ends of filaments.They are the most widely distributed group of microorganisms in nature and arewell known as saprophytic soil inhabitants (Takizawa *et al*., 1993). They have the important roles in soil biodegradation and recycling of nutrients associated with recalcitrant polymer. They can degrade an enormous number and varieties of organic compounds are extremely important in the mineralization of organic matter. In natural habitats, genus *Streptomyces* exist a major component of the total actinomycetes population(Suzuki *et al*., 2000). Actinomycetes represent a high proportion of the soil microbial biomass and have the potential to produce a diverse range of secondary metabolites including various antibiotics, anticancer and immunosuppressive agents and plant growth hormones (Strobel and Daisy, 2003; Fiedler *et al*., 2008; Schulz *et al.,* 2009) that play an important role in agriculture and pharmaceutical industry. Actinomycetes have been proven to be a rich source of important natural products especially antibiotics. More than 22,000 biological active compounds have been obtained from microbes by the end of 2002, among them, 45% were produced by actinobacteria, especially by genus *Streptomyces* (Berdy, 2005).

Endophyte is anorganism, which reside the whole or part of its life cyclecolonizing inter- and/or intra-cellularly inside the healthy living tissues of thehost plant, without causing apparent symptoms of disease(Schulz and Boyle, 2006). Endophytes colonizing inside plants and usually get nutrition and protection from the host plants. In return, endophytes profoundly enhanced the health of the host plants by helping plants in nutrition supply and by producing a variety of bioactive metabolites(Nishimura *et al*., 2006).

It is noteworthy that, of the nearly 300,000 plant species on the earth, each individual plant is considered to host one or more type of endophytes (Strobel and Daisy, 2003). Endophytes represent a subgroup of the rhizobacterial communities which may have the ability to enter

the roots of their host after therhizosphere is colonized (Rosenblueth and Martinez Romero, 2006). Endophytic actinomycetes are the microbes that reside in healthy tissues of living plants without causing clinically detectable symptoms of disease and can be isolated from the surface-sterilized plant tissues (Nimnoi and Pongslip, 2009). These microbes live in different plant organs like roots, stems, leaves, petioles) of the host plants, mainly in inter or intracellular spaces.As a result of these long-held associations, endophytic microorganisms and plants have developed good information transfer systems (Strobel, 2003). Endophytic actinomycetes are increasingly important, because they have the ability to produce the bioactive compounds inhibiting some of the pathogenic fungi and bacteria (Sardi *et al*., 1992).

A variety of actinomycetes inhabit a wide range of plants as symbionts, parasites or saprophytes were reported and most of them belong to the genus*Streptomyces* and *Microbispora*(Matsumoto *et al.,* 1998). In general, *Streptomyces* sp. was the most predominant species and *Microbispora*, *Micromonospora*, *Nocardioides*, *Nocardia* and *Streptosporangium* are the common genera. *Streptomyces*was an excellent producer of bioactive metabolites and serve as an important source for the discovery of novel bioactive products (Ryan*et al.,* 2008).Besides *Streptomyces* sp., other genera like *Tsukamurella* and *Corynebacterium* isolated from *Maytenus austroyunnanesis* (Qin *et al.,* 2012),*Actinomycete* sp. from *Mirabilis jalapa*(Golinska *et al*., 2015) *Leifsonia* from Ginseng roots (Qiu *et al.,* 2007) and *Brevibacterium* isolated from *Centella asiatica* and *Conyza canadensis* (Kim *et al*., 2012) were also reported as rare endophytic actinomycetes from medicinal plants.There are very few recent reports regarding the microbial studies on the endophytic actinomycetes residing in the traditional medicinal plants. Medicinal plants are a potent source of endophytic actinomycetes with broad biological actions against pathogenic fungi as well as Gram positive and negative bacteria (Wu, 2006). Thus, this habitat deserves close examination for potential and novel microbes that could produce compounds with desired bioactivities (Gangwar *et al.,* 2014). Endophytes of medicinal plants probably participate in metabolic pathways of medicinal plants and produce analogous or novel bioactive compounds, for example, taxol (Strobel *et al.,* 1999). Zhao *et al.* (2011) screened the endophytic actinomycetes of medicinal plants from Panxi plateau based on the medicinal function of the plants to identify their potential as biocontrol agents for phytopathogens and bacteria. A large population of plant growth promoting microbes is found both in rhizosphere and inside plants (Bhosale and Kadam, 2015).Endophytic actinomycetes have

attracted attention in the search for novel bioactive natural compounds that can be used as new drugs replacing those against which pathogenic strains have rapidly acquired resistance. Actinomycetes are the main source of antibiotics and endophytic actinomycetes isolated from medicinal plants has considerable developed this potential (Priya, 2012). Many endophytic actinobacteria, especially those from medicinal plants possess the ability of inhibiting a wide variety of harmful microorganisms like pathogenic bacteria, fungi and viruses (Qin *et al.,* 2011). The association of actinomycetes with plants is found to confer many advantages such as the production of antimicrobials, extracellular enzymes, phytohormone and siderophores. They also help in phosphate solubilization and plant protection against abiotic and biotic stresses (Bailey *et al.,* 2006; Clegg and Murray, 2000). They are also known for their ability to promote plant growth by producing plant growth hormone such as indole acetic acid, nitrogen fixation, phosphate solubilization and siderophore production (Cattelan *et al*. 1999; Franco-Correa *et al.* 2010). Hence, they have been demonstrated to improve and promote growth of host plants as well as to reduce disease symptoms caused by plant pathogens or various environmental stresses (Hasegawa *et al.,* 2006).

North Eastern Region of India is a big bioprospecting area and best known for its rich biodiversity and un-tapped bioresources which has been identified as a significant portion of both the Himalaya and Indo-Burma biodiversity hotspots (Myers et al., 2000). Mizoram is an important state of Northeastern (NE) India and also is a part of the 25 mega-biodiversity hotspots of the world. It has been well documented that medicinal plants with an ethnobotanical history are high in diversity. There are reported of more than 200 ethnomedicinal plants for their efficiency to cure various diseases (Lalramnghinghlova and Jha, 1998). A categorical list of plant species of 159 ethnomedicianl plant species belonging to 134 genera and 56 families recorded from tropical forests, home gardens, roadsides and University Campus of Mizoram have been described by (Rai and Lalramnghinglova, 2010).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 endophytic actinomycetes in these region remains unexplored and uncharacterized. Moreover, report of studies on the genetic diversity and plant growth promoting of endophytic actinomycetes of these area are scanty. Recently, reported few studies on endophytic actinomycetes having biosynthetic potential and plant growth promoting abilities (Passari *et al.,* 2015a and b).Therefore, it is very important to study and preserve the genetic diversity associated with medicinal plants in this region to

understand the role of endophytic actinomycetes in plants.To understand the diversity of endophytic actinomycetes and search for noble antimicrobial agents for sustainable agriculture from traditionally used medicinal plants following objectives were kept for this study.

- Identification and invitro antagonistic activities of endophytic actinomycetes associated with some ethno-medicinal plants of Mizoram.
- Screening of potential antagonistic isolates for plant growth promoting traits.
- Phylogenetic analysis of antagonistic isolates by using REP-PCR (BOX-PCR and ERIC-PCR) markers.

CHAPTER 2

REVIEW OF LITERATURE

Endophytic actinomycetes/actinobacteria are considered as potential sources of novel bioactive compounds and various compounds have been isolated from them until now (Taechowisan *et al.*, 2005; Igarashia *et al.*, 2007). Endophytic actinobacteria have been isolated from a variety of healthy plant species ranging from crop plants, such as wheat, rice, potato, tomato and citrus (Coombs and Franco, 2003; Tian *et al*., 2007),different woody tree species (Taechowisan *et al.*, 2003; Zin *et al.*, 2007; Zhao *et al.*, 2010a, b, c) and ferns and club mosses (Janso and Carter, 2010). Among endophytic actinomycetes genus *Streptomyces* was found most frequently in nature. For instance, 619 actinomycetes were isolated from different cultivars of tomato, and all of them were *Streptomyces* sp. (Tan *et al.*, 2006). From 36 medicinal plant species of Thailand,Taechowisan *et al.*, (2003) isolated 330 strains belonging to four different genera (*Streptomyces*, *Microbispora*, *Nocardia* and *Micromonospora*) and reported that endophytic actinomycetes, especially *Streptomyces* sp. were the most common isolates recovered, being most prevalent from roots, leaves and less from stems. Lee *et al.*, (2008) isolated 81 endophytic actinobacteria including eight genera from Chinese cabbage roots, and *Microbispora* sp. were the most common isolates, followed by *Streptomyces* sp. and *Micromonospora* sp. Endophytic actinomycetes have been isolated from stem and root interior of many plants, such as snakevine, tomato, banana, neem, wheat, etc (Cao*et al*., 2004, 2005; Verma *et al.*, 2009; Coombs and Franco, 2003).Actinomycetes genus especially *Streptomyces* sp. have long been recognized as prolific producer of useful bioactive metabolites with broad spectrum of activities, such as antibacterial, antifungal, antibiotic, antiparasitic, antitumor, antiviral, insecticide, herbicide, immunomodulators, antithrombotic agents (Atta and Ahmad , 2009; Baltz, 2008). *Streptomyces* alone cover about 80% of total antibiotic products as compared to other actinomycetes genera (Keiser *et al*., 2000) and providing more than half of the naturally occurring antibiotics discovered to date and continuing to be a major source of many types of antibiotics and other class of biologically active secondary metabolites (Okamoto *et al*., 2003).

Growth of microbes in the laboratory is dependent on the composition of the media and the cultivation conditions that are applied. For endophytic actinobacteria, some classical media

for soil actinomycetes isolation, such as humic acid vitamin (HV) (Hayakawa, 1990), International *Streptomyces* Project media ISP 2 and ISP 5 (Shirling and Gottlieb 1966)and starch casein agar (Kuster and Williams 1964) are well known. Likewise, Low nutrient medium TWYE was found effective for isolation of endophytic actinobacteria (Coombs and Franco 2003; Qin *et al*., 2009b; Li *et al*., 2009a), due to the fact that high nutrient concentration allowed fast growing bacteria to overgrow slower growing actinobacteria. Tryptic Soy Agar (TSA) were used and morphological characters of endophytic actinomycetes was observed (Gangwar *et al.,* 2014). The endophytic actinobacterial communities are diverse and the extent of diversity may vary between different sample collection regions and different plant species. The diversity of genera and the number of culturable endophytic actinobacteria was largely dependent on the isolation method (Qin *et al.,* 2011).

Several endophytic actinomycetes have been reported as potential biocontrol agents that may improve and promote plant health. Isolated and identified a total of 17 actinomycetes from Karangkadu mangrove ecosystem in the leaves of 5 different halophytic plants such as *Avicennia marina, Bruguiera cylindrica, Rhizophora mucronata, Salicornia brachiata* and *Suaeda monoica* and observed that few of the endophytic actinomycetes have persistent antibacterial activity (Ravikumar *et al*., 2011.). Twelve actinomycetes strains isolated from *Taraxicum officinale* rhizosphere were active against *Pythiumultimum*(Crawford *et al.* (1993). *Streptomyces rochei* and *S. rimosus* from the chickpea rhizosphere were strong antagonists of *Fusarium oxysporum* (Bashar and Rai, 1994). Ouhdouch *et al.,* (2001) found 10 isolates of actinomycetes from medicinal plant rhizosphere soils, most of which were *Streptomyces* sp. After testing for antifungal activity against *Candida albicans* and *C. tropicalis,* they found that all *Streptomyces* had antifungal activity. Thangapandian *et al.* (2007) isolated *Streptomyces* from medicinal plant rhizosphere soils and 8 isolates had antipathogenic activity and six strains of 10 *Streptomyces* and *Micronomospora* isolated from rhizosphere soil of *Vitis vinifera* L. produced antifungal metabolites strongly inhibited *Botrytis cinerea* (gray mold of grapevines) (Loqman *et al.,* 2008). The endophytic *Streptomyces* NRRL 30562 obtained from the snakevine produced novel peptide antibiotic with wide-spectrum activity against many pathogenic fungi and bacteria (Castillo *et al.,* 2002). *In vitro* antifungal activity was determined using the dual culture bioassay against four fungal phytopathogens, *Fusarium solani, Phytophthora infestans, Macrophomina phaseolina, Rhytisma acerinum* and *Botrytis cinerea*, which are the causative agents of tomato's wilt,

potato's late blight, soybean's charcoal rot, tar spot disease and eggplant fruit rot, respectively. Out of the 47 isolates, *Streptomyces sp. 5* and *Streptomyces sp.7* were prolific producers of fungal inhibitory bioactive compounds. Both isolates were investigated for the production of antifungal diffusible compounds, volatile compounds and enzymes (chitinase, cellulose, and CMCase). They showed *in vitro* activity against the five previously mentioned fungal pathogens as well as another pathogen (*Fusarium oxysporum*), with a probability of $p<0.05$. The competitive growth between pathogens and antagonists on solid media revealed lesser suppression, comparing to that recorded for the diffusates on agar. Additionally, greater antagonistic effects were recorded for crude metabolites, membrane diffusible metabolite(s), and volatiles, respectively. However, both actinomycetesisolate showing high ability to produce chitinases and cellulases. The potential of using *Streptomyces sp.* 5and *Streptomyces* sp.7as phytopathogen biocontrol agents was discussed (Priya, 2012).

Generally, plant growth promoting rhizobacteria (PGPR) promote plant growth directly by either facilitating resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents (Kloepper and Schroth, 1981).

Endophytic actinomycetes are also known for their ability to promote plant growth by producingplant growth hormone such as indole acetic acid, nitrogen fixation, phosphate solubilisationand siderophore production (Cattelan *et al.* 1999; Franco-Correa *et al.* 2010). IAA is a commonly natural compound which is a product of L-Tryptophan metabolism in microorganisms. Auxin represent wide group of the derivatives of indole ring compounds. This compound has the ability to improve plant growth by stimulating cell elongation, root initiation, increase seed germination and seedling growth (El-Tarabily, 2008). Three medicinal plants, *Aloe vera*, *Mentha arvensis* and *Ocimum* sanctum were explored for endophytic actinomycetes diversity, plant growth promoting and antimicrobial activity, where recovered isolates most commonly genus *Streptomyces* sp. frequently found from roots showed good amount of plant growth promoting activities (Gangwar *et al*., 2014). Khamna *et al.* (2010) isolated a collection of *Streptomyces* spp. from the rhizosphere soils of 14 Thai medicinal plants, out of which 11.2% were found to produce indole-3-acetic acid in a yeast malt extract medium supplemented with 2 mg/ml L-tryptophan. There are many reports which demonstrated the ability of endophytic *streptomycetes* to produce indole acetic acid

and thus promote plant growth (Tsavkelova *et al.* 2006; Solans *et al.* 2011). A total of 252 endophytic actinomycetes isolates were recovered from mandarin(*Citrus reticulata* L.). Based on spore chain morphology, cell wall type, and 16S rRNA gene sequence, the isolates were classified into six genera: *Streptomyces*, *Nocardia*, *Nocardiopsis*, *Spirillospora*, *Microbispora* and *Micromonospora.* The most frequent isolates recovered were members of *Streptomyces* (85.3%). Selected isolates (64 isolates) from these genera were evaluated for their indole-3-acetic acid (IAA) production potential in a medium with 2 mg mL−1 tryptophan, and all the selected isolates showed the potential to produce IAA. Isolates of genus *Nocardiopsis* showed a very high ability to produce IAA that was the highest among all the genera, with values ranging from 62.23 to 222.75 μg mL−1. Twelve isolates selected from these genera were inoculated onto mandarin seedlings, and the results indicated that the shoot height, fresh shoot weight and fresh root weight of the seedlings were promoted by the inoculation of endophytic actinomycetes, with values ranging from 20.2 to 49.1%, 14.9 to 53.6%, and 1.6 to 102% over the control, respectively (Shutsrirung *et al*., 2013).

Therefore, the association of actinomycetes with plants is found to confer many advantages such as the production of antimicrobials, extracellular enzymes, phytohormones and siderophores. They also help in phosphate solubilization and plant protection against biotic and abiotic stresses (Bailey *et al*., 2006; Clegg and Murray, 2000).

The application of genetic approaches, especially 16S rRNA gene analysis, to actinomycetes classification has contributed considerably to our understanding of the phylogenetic relationships between among genus. The applications of 16S rRNA gene-based culture independent molecular approaches, such as polymerase chain reaction (PCR)-based 16S rRNA gene clone library, denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphism (T-RFLP) analysis are useful to reveal the complex microbial community inhabiting various plants. Sometimes, the combination of culturing methods and culture-independent analysis is needed for the study of endophytic community (Qin *et al*., 2011).

More recently, a molecular technique, denaturing gradient gel electrophoresis (DGGE), has been employed to investigate microbial communities within different plant organs and different plant rhizospheres in many ecological systems (Das *et al*., 2007) and (Prakamhang

et al., 2009). Using this technique, it ultimately leads to understand the ecology of these microorganisms that are important to be studied in their natural habitats.

BOX-PCR is based on the observation that outwardly facing oligonucleotide primers, complementary to interspersed repeated sequences, enable the amplification of differently sized DNA fragments, consisting of sequences lying in between these elements. It has been applied in numerous taxonomic studies on plant-associated, environmental, medical and food-associated bacteria (Lanoot *et al*., 2004).

RAPD technique was applied extensively in studying bacteria; (Williams *et al*., 1990) demonstrated the usefulness of RAPD in differentiating between bacterial genera. Applying RAPD to *streptomycetes* was first done by (Mehling *et al*., 1995) when they used various arbitrary primers to locate RAPD markers. A 1100 bp band was identified and appeared in all actinomycetes tested. Hybridization experiments showed that this band is useful in identification of this bacterial group. The interspecific, intraspecific and intraclonal polymorphisms between *Streptomyces* strains using RAPD methodology was recently used for the rapid, sensitive and specific detection of genetic diversity among species and strains of *Streptomyces* (Martin *et al*., 2000). Recently, natural products screening was focused on the discovery of bioactive metabolites, especially the discovery and identification of their genetic information. As complete sequences of many biosynthetic gene clusters related to different kinds of compounds have been gained, it is possible to detect genes involved in the synthesis of secondary metabolites to evaluate the biosynthetic potential (Ayuso-Sacido and Genilloud, 2004), e.g., 3-amino-5-hydroxybenzoic acid synthase (AHBA), cytochrome P450 hydroxylases (CYPs) and epoxidase (ES) are needed for ansamycin, polyene and polyether antibiotics biosynthesis, respectively.

CHAPTER 3

MATERIALS AND METHODS

3.1. Plant collection and description

Five healthy medicinal plants, viz. *Senecio scandens, Costus speciosus, Ageratum conyzoides, Mikania micrantha and Cassia fistula* were collected from Murlen National Park (23°37″N; 93°18″E),Mizoram, Dampa Tiger Reserve Forest(23°25′N; 92°20′E)and Mizoram University (MZU) Campus (21°58' and 24°35'N and 92°15' and 93°29'E), Mizoram, India during November, 2014 (Table 3.1& Figure 3.1 and 3.2). The selection of plant species was based on their ethanobotanical history and their abundance. The tissues from root, stem, leaf and petiole was removed carefully to ensure that the sufficient amount of tissues was collected. All the materials were placed in sterile polythene bags and brought into the laboratory in an icebox and processed within 24 h of collection.

Table 3.1 Summary of plant sample collection from three locations, their taxonomic status and traditional medicinal uses.

Voucher number	Scientific name	Local name	Family	Habit	Medicinal uses and References
MZU/BT/014	*Ageratum conyzoides*	Vailenhlo	Asteraceae	Herb	Stomach cancer and anti-diarrhoeal (Rai and Lalramghinglova, 2010).
MZU/BT/015	*Mikania micrantha*	Japanhlo	Asteraceae	Herb	Dysentry, fever, cuts and wounds (Rai and Lalramnghinghinglova, 2010).
MZU/BT/016	*Senecio scandens*	Saiekhlo	Asteraceae	Climber	Cancer/ ulcers (Rai and Lalramnghinglova, 2010)
MZU/BT/017	*Costus speciosus*	Sumbul	Costaceae	Creeper	Kidney problem (Lalmuanpuii et al., 2013)
MZU/BT/018	*Cassia fistula*	Makpazangkang	Fabaceae	Tree	Skin infections, fever, stomach problems

Fig3.1: Map of study areas showing the collection places

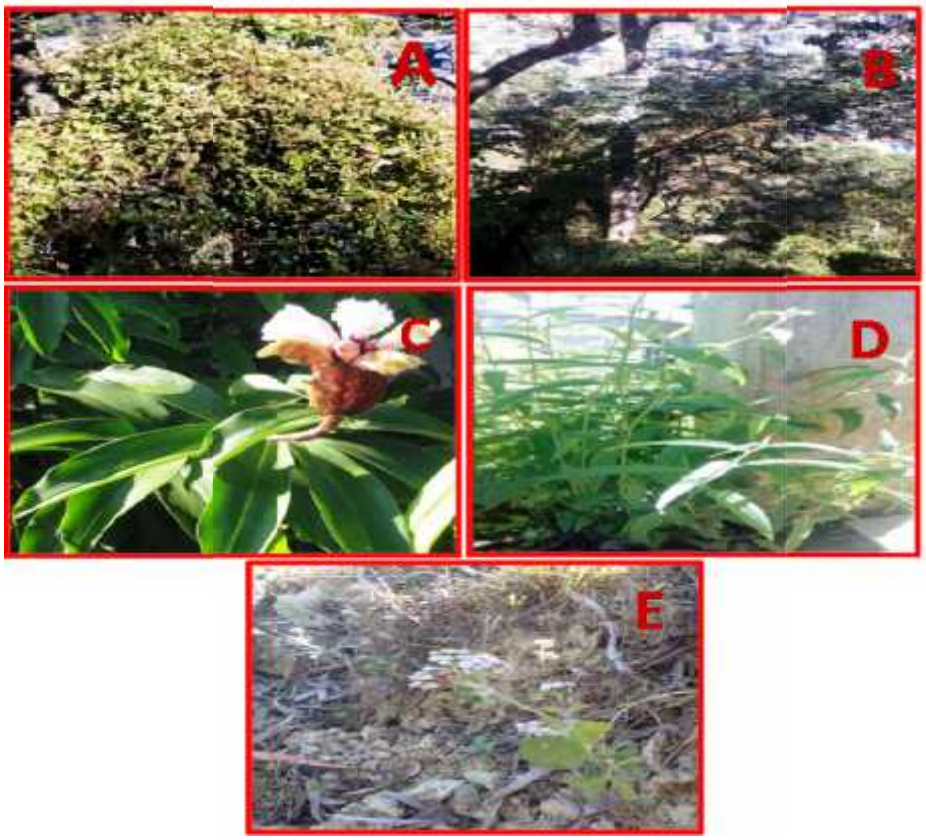

Fig 3.2: Five different plants of the present study.A- *Senecio scandens;* B-*Costus speciosus;* C-*Cassia fistula;* D- *Mikania micrantha and* E- *Ageratum conyzoides.*

3.2. Isolation of endophytic actinomycetes

Different plant parts (leaf, stem, rootand petiole) was used for the isolation of endophytic actinomycetes by using five different media (SCNA, SCA, AIA, TH_2O and ISP5) as described by Taechowisan and Lumyong (2003). The media were supplemented with Nalidixic acid to suppress bacterial growth and Cycloheximide (100mg/L) to suppress fungal growth. The plates were incubated at 28±2^{o}C in BOD incubator for up to 4 weeks. The plant segments were observed once a day for the growth of endophytic actinomycetes.

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 pigments, spore chain morphology etc. was studied by (Thampayak *et al*., 2008). The spore chain morphology and surface of spore were examined by Field Emission Gun—Scanning electron microscopy (FEG-SEM) of 10-day old cultures grown on ISP1 media. The organism was identified by following the keys of Bergey's Manual of Determinative Bacteriology (Bergey and Holt, 2000).

3.4. Screening for *In vitro* antagonistic potential of endophytic actinomycetes isolates

All the isolates were screened for their antagonistic activity against four major plant pathogenic fungi i.e. *Fusarium oxysporum* f. sp. *ciceri* (MTCC-2791), *Fusarium proliferatum* (MTCC-286), *F. Graminearum* (MTCC-1893) and *Fusarium udum* (MTCC-2755) by dual culture *in vitro* assay (Bredholdt *et al.,* 2007). The pathogens were obtained from Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India. Plates with only pathogen culture were served as control. All plates was incubated at 28 ^{0}C and percentage of inhibition was calculated by using the formula C-T/C x 100, where, C is the colony growth of fungal pathogen in control, and T is the colony growth in dual culture. All experiments were carried out in triplicates and mean was recorded.

3.5. Screening for phosphate solubilization of endophytic actinomycetes

Qualitative phosphate solubilization activity of potential antagonistic endophytic actinomycetes isolates was carried out according to the method of (Doumbou *et al.* 2001). Endophytic actinomycetes isolates were inoculated on Pikovskaya medium and incubated at

28°C for seven days. The halo zone around the colony was presumptive confirmation of phosphate solubilization.

3.6. Production of indole-3-acetic acid of endophytic actinomycetes

The production of IAA by endophytic actinomycetes isolates was estimated according to Gordon and Weber (1951). 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 color and the absorbance will be measured at 530 nm using a spectrophotometer and compared with the standard curve of IAA and the amount of IAA was expressed in µg/ml.

3.7. Production of ammonia of endophytic actinomycetes

The endophytic actinomycetes isolates was tested for the production of ammonia 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. A 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 The absorbance was measured at 480 nm using a spectrophotometer and compared with the standard curve of $(NH_4)_2SO_4$ and expressed in mg/ml.

3.8. Screening for catalase production of endophytic actinomycetes

A loop full of test culture was transferred to a sterile glass slide and a drop of 3% hydrogen peroxide (H_2O_2) was added to the culture at once and observed for effervescence. Evolution of effervescence indicated catalase production (Singh and Padmavathy, 2014).

3.9. Cellulase production of endophytic actinomycetes

Screening of cellulase producers was done according to the method of Kasana *et al*. (2008). Suitable broth was prepared for each positive strain and was inoculated with the strain. After optimal growth is achieved and then centrifuged at 5,000 rpm for 10 min. The cell-free supernatant (crude enzyme extract) was collected and enzymatic assay was performed. 0.5ml of suitable substrate (1% CMC) was added to 0.5 ml of enzyme solution concentrated or diluted in respective buffer to 1ml and incubated for 20-50 min at 55°C. The reaction was

stopped by the addition of 3ml of DNS (dinitro salicylic acid) reagent and boiled for 5 to 10 min. Soon after 1ml of 40% Potassium Sodium Tartarate was added. On cooling, the absorbance was taken at 540 nm. 1 unit (IU) is defined as the amount of enzyme that released 1µmol of glucose from CMC per minute at standard conditions and their quantification assay was done according to the method mentioned earlier (Kavya *et al*., 2012; Ghose *et al*., 1987).

3.10. Amylase production of endophytic actinomycetes

Screening of amylase production was done by using the method of (Kasana *et al*., 2008). Suitable broth was prepared and inoculated each positive strain. After optimal growth is achieved and then centrifuged at 5,000 rpm for 10 min. The cell-free supernatant (crude enzyme extract) was collected and enzymatic assay was performed. 0.5ml of suitable substrate (1% starch) was added to 0.5 ml of enzyme solution concentrated or diluted in respective buffer to 1ml and incubated for 20-50 min at 37-55 ^{0}C. The reaction was stopped by addition of 3ml of DNS (dinitro salicylic acid) reagent and boiled for 5 to 10 min. Soon after 1ml of 40% Potassium Sodium Tartarate was added. On cooling the reaction mixture; the absorbance was read at 540 nm. 1 unit (IU) is defined as the amount of enzyme that released 1µmol of glucose from CMC per minute at standard conditions and their quantification assay was followed the method of (Mohan and Charya, 2012).

3.11. Molecularcharacterization and phylogenetic analysis of endophytic actinomycetes

3.11.1. Genomic DNA extraction, amplification of 16S rRNA gene and sequencing

Total genomic DNA was extracted by DNA extraction Kit (invitrogen). The DNA purified and quantified by absorption spectrophotometry at 260 and 280. 16S rRNA gene sequence was amplified by using universal primers-PA: 5'-AGAGTTTGATCCTGGCTCAG-3' as the forward primer and PH: 5'-AAGGAGGTGATCCAGCCGCA-3' as the reverse primer (Qin *et al*., 2009). The PCR amplification were carried out under the following conditions: initial denaturation at 95˚C for 4 min, followed by 30 cycles of denaturation at 94˚C for 1 min, annealing at 57˚C for 1 min and extension at 72˚C for 1.2 min with a final extension step at 72˚C for 10 min. Negative controls (no added of DNA) were included in all sets of reactions. The reactions were performed in a Veriti thermal cycler (Applied Biosystem, Singapore) contained a total volume of 25µl consisting of 1.0µl genomic DNA (50ng), 0.5µl of each primer (10 pmol), 2.0µl deoxynucleotide triphosphates (2.5 mM each), 2.5µl of 1X PCR buffer, 1.0µl of Taq DNA polymerase (1U/µl) and 17µl MilliQ grade water. After PCR

amplification,amplified PCR product was determined by 1% (w v^{-1}) agarose gel electrophoresis and were sequenced at SciGenome Pvt. Ltd. Kochin, India.

3.11.2. BOX-PCR fingerprinting of endophytic actinomycetes

DNA amplification was performed as per the procedure of (Rademaker *et al*., 1998) using the BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3'). Amplified PCR fragments was separated on 2.0 % (w/v) agarose gels. Post electrophoresis staining of gel was done in 0.5 mg of ethidium bromide per ml and after distaining, the gels were photographed. Calculation of the similarity of PCR fingerprinting profiles was made on the basis of the Pearson product-moment correlation coefficient. A dendrogram was produced from the matrix of similarities by the unweighted pair group method using arithmetic average (UPGMA) clustering algorithm.

3.11.3. ERIC-PCR fingerprinting of endophytic actinomycetes

The PCR reactions was carried out as described by (Versalovic *et al.,* 1991) using a set of primer sequences ERIC-1R (5′-CACTTAGGGGTCCTCGAATGTA- 3′) and ERIC-2F (5′-AAGTAAGTGACTGGGGTGAGCG- 3′) to amplify the regions in the bacterial genome positioned between the ERIC sequences. The amplified products was separated by electrophoresis on 1.5% agarose gel using 1x TAE buffer. The PCR bands was analyzed under UV light and documented using a BioRad Gel Doc XR^{+} system (Hercules, CA, USA).

3.11.4. Phylogenetic analysis of endophytic actinomycetes

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 was pairwise aligned using the program Clustal W packaged in the MEGA 4.0. software (Thompson *et al*., 1997). The data obtained was used to derive phylogenetic tree with the same softwere and a Neighbour Joining tree and Maximum likelihood was generated (Saitou and Nei, 1987). Bootstrap analyses with 5,000 resamplings was performed with MEGA 4.0using p-distance model (Felsenstein, 1985).

Polymorphic DNA fingerprints was scored in the binary form i.e. 1 for presence of a band and 0 when there is absence band, to generate a binary matrix for ERIC and BOX markers. The binary matrix will be used to calculate the Simple Matching (SM) coefficient;

phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method supported by Numerical Taxonomy SYStem (NTSYS version 2.2).

CHAPTER 4

RESULTS

4.1. Isolation of endophytic actinomycetes

In total, 87 strains of endophytic actinomycetes were isolated from root, stem, leaf and petiole of five selected traditional medicinal plants collected from Dampa Tiger Forest Reserve, Murlen National Park and Mizoram University Campus. We obtain 30 (34.4%) isolates from Mizoram University Campus, 20 (22.9%) from Murlen National Park and 37 (42.5%) from Dampa Tiger Forest Reserved. According to the morphological, cultural and microscopic characteristics (Table 4.1) (Fig 4.3 and 4.4), we noted that *Streptomyces* was the dominant species and majority of the isolates were recovered from the roots (n=40, 45.8%) followed by stems (n=22, 25.4%), leaves (n=15, 17.2%) and petioles (n=10, 11.4%) (Fig 4.1). Based on media composition, we obtained 30 isolates from Starch Casein Agar (SCA), 27 isolates from Starch Casein Nitrate Agar (SCNA), 25 isolates from International *Streptomyces* Project 5 (ISP5), 3 isolates from Tap Water Yeast Extract Agar (TWYE) and only 2 isolates from Actinomycetes Isolation Agar (AIA) (Fig 4.1). Out of five selected plants, 30 isolates was obtained from *Mikania micrantha*, 25 isolates from *Ageratum conyzoides*, 15 isolates from *Costus speciosus*, 10 isolates from *Senecio scandens* and 7 isolates from *Cassia fistula* (Table 4.2).

Relative abundance of endophytic actinomycetes at the genus level reveals that *Streptomyces* was most abundant at root tissue with 92.5%. However, some rare isolates like *Nocardiopsis*, *Tsukamurella* and *Actinobacteria*was found only in root tissue with 2.5% each. Only *Streptomyces* was foundin leaf and petiole parts with 100%. These results indicate that endophytic actinomycetes were highly dominant in root followed by stem, leaf and petiole and the population of endophytic actinomycetes varies between different climate conditions.

Table 4.1: Morphological characteristics of endophytic actinomycetes isolates

Isolate no.	Isolate Identified sp.	Growth and colony nature	Aerial Mycelia	Substrate Mycelia	Pigmentation
BPSEAC1	*Streptomyces* sp.	Slow and sticky	White	White	Green
BPSEAC2	*Streptomyces* sp.	Slow and rough	White	Orange	-
BPSEAC3	*Nocardiopsis* sp.	Slow and powdery	Cream	Brown	-
BPSEAC4	*Streptomyces* sp.	Slow and sticky, colony with ring	Cream	Cream	Light yellow

BPSEAC5	*Streptomyces thermocarboxudus*	Slow and sticky	Dark grey	Dark brown	-
BPSEAC6	*Streptomyces* sp.	Slow and rough	Cream	Brown	-
BPSEAC7	*Streptomyces* sp.	Slow and sticky	Cream	Cream	-
BPSEAC8	*Streptomyces* sp.	Slow and rough	White	Grey	-
BPSEAC9	*Streptomyces* sp.	Slow and rough	Off-white	Cream	-
BPSEAC10	*Streptomyces* sp.	Slow and rough	Cream	Yellow	-
BPSEAC11	*Streptomyces olivaceus*	Slow and rough	Off-white	Cream	-
BPSEAC12	*Streptomyces* sp.	Slow and powdery	Cream	Orange	-
BPSEAC13	*Streptomyces* sp.	Slow and powdery	Cream	Orange	-
BPSEAC14	*Streptomyces* sp.	Slow and sticky and hard	Dark grey	Grey	-
BPSEAC15	*Streptomyces olivaceus*	Slow and rough, 10mm colony size	Yellow	Cream yellow	-
BPSEAC16	*Streptomyces olivaceus*	Slow and sticky,	Orange	Orange cream	-
BPSEAC17	*Streptomyces* sp.	Slow and rough	Cream	Orange	-
BPSEAC18	*Streptomyces olivaceus*	Slow and sticky	White	Cream	Light yellow
BPSEAC19	*Streptomyces bikinensis*	Slow and sticky	Light orange	Brown	-
BPSEAC20	*Streptomyces* sp	Slow and sticky	Light orange	Brown	-
BPSEAC21	*Streptomyces* sp	Slow and rough	Light brown	Light brown	-
BPSEAC22	*Streptomyces olivaceus*	Slow and sticky, small colony	White	Cream	-
BPSEAC23	*Streptomyces olivaceus*	Slow and sticky	Cream	Yellow	-
BPSEAC24	*Streptomyces prasinipilous*	Slow and sticky	White	Yellow brown	-
BPSEAC25	*Streptomyces* sp	Slow and rough	White	Cream	-
BPSEAC26	*Streptomyces* sp	Slow and rough			-
BPSEAC27	*Streptomyces* sp	Slow and rough	White	Cream	-
BPSEAC28	*Streptomyces albogriseoplanus*	Slow and rough	Dark grey	Light grey	-
BPSEAC29	*Streptomyces qriseoplanus*	Slow and rough	White	Cream	-
BPSEAC30	*Streptomyces* sp	Slow and smooth	Dark grey	Light grey	-
BPSEAC31	*Tsukamurella* sp.	Slow and rough	White	Grey	-
BPSEAC32	*Streptomyces* sp	Slow and rough	White	Cream	-

BPSEAC33	*Streptomyces* sp	Slow and rough	White	Cream	-
BPSEAC34	*Streptomyces qriseoplanus*	Slow and rough	Grey	Green brown	-
BPSEAC35	*Streptomyces olivaceus*	Slow and rough	Cream white	Yellow	-
BPSEAC36	*Nocardiopsis* sp.	Slow and smooth	Cream	Brown	-
BPSEAC37	*Streptomyces* sp	Moderate and rough	White	White-cream	-
BPSEAC38	*Streptomyces* sp	Moderate and sticky	White	White-cream	-
BPSEAC39	*Streptomyces olivaceus*	Slow and rough	White	Orange	-
BPSEAC40	*Streptomyces* sp	Moderte and smooth	Cream	Brown	-
BPSEAC41	*Streptomyces albidoflavus*	Moderate and smooth	White	White-cream	-
BPSEAC42	*Streptomyces specialis*	Slow and smooth	Cream	Yellow	-
BPSEAC43	*Actinobacteria bacterium*	Moderate and powdery	Cream	Light yellow	-
BPSEAC44	*Streptomyces violascens*	Moderate and smooth	White	White-cream	-
BPSEAC45	*Streptomyces* sp	Slow and hard, small colony	White	White grey	-
BPSEAC46	*Streptomyces* sp	Moderate and hard	White	Orange	-
BPSEAC47	*Streptomyces* sp	Slow and powdery	Cream	Cream	-
BPSEAC48	*Streptomyces* sp	Slow and rough	White	Cream	-
BPSEAC49	*Streptomyces* sp	Slow and rough	Grey	Green brown	-
BPSEAC50	*Streptomyces* sp	Slow and rough	Cream white	Yellow	-
BPSEAC51	*Streptomyces* sp	Slow and hard	Cream	Brown	-
BPSEAC52	*Streptomyces* sp	Slow and rough	White	White-cream	-
BPSEAC53	*Streptomyces* sp	Slow and hard	White	White-cream	-
BPSEAC54	*Streptomyces* sp	Moderate and sticky	White	Orange	-
BPSEAC55	*Streptomyces* sp	Slow and rough	Cream	Brown	-
BPSEAC56	*Streptomyces* sp	Moderate and smooth	White	White-cream	-
BPSEAC57	*Streptomyces* sp	Slow and rough	Grey	Green brown	-
BPSEAC58	*Streptomyces* sp	Slow and smooth	Cream white	Yellow	-
BPSEAC59	*Streptomyces* sp	Slow and rough	Cream	Brown	-
BPSEAC60	*Streptomyces* sp	Slow and rough	White	White-cream	-
BPSEAC61	*Streptomyces* sp	Slow and rough	White	White-cream	-
BPSEAC62	*Streptomyces* sp	Slow and rough	White	Orange	-
BPSEAC63	*Streptomyces* sp	Slow and rough	Cream	Brown	-

BPSEAC64	*Streptomyces* sp	Slow and powdery	White	White-cream	-
BPSEAC65	*Streptomyces* sp	Slow and rough	White	Grey	-
BPSEAC66	*Streptomyces* sp	Moderate and smooth	White	Cream	-
BPSEAC67	*Streptomyces* sp	Slow and sticky	White	Cream	-
BPSEAC68	*Streptomyces* sp	Slow and sticky	White	Cream	-
BPSEAC69	*Streptomyces* sp	Slow and sticky	White	Cream	-
BPSEAC70	*Streptomyces* sp	Slow and sticky	Cream	Orange	-
BPSEAC71	*Streptomyces* sp	Slow and smooth	Grey	Off-white	-
BPSEAC72	*Streptomyces* sp	Slow and powdery	White	White-cream	-
BPSEAC73	*Streptomyces* sp	Slow and rough	White	White-cream	-
BPSEAC74	*Streptomyces* sp	Slow and smooth	White	Orange	-
BPSEAC75	*Streptomyces* sp	Slow and rough	Cream	Brown	-
BPSEAC76	*Streptomyces* sp	Slow and rough	White	White-cream	-
BPSEAC77	*Streptomyces* sp	Slow and rough	White	White-cream	-
BPSEAC78	*Streptomyces* sp	Slow and rough	White	Orange	-
BPSEAC79	*Streptomyces* sp	Slow and rough	Cream	Brown	-
BPSEAC80	*Streptomyces* sp	Slow and rough	White	White-cream	-
BPSEAC81	*Streptomyces* sp	Slow and powdery	Grey	Green brown	-
BPSEAC82	*Streptomyces* sp	Slow and rough	Cream white	Yellow	-
BPSEAC83	*Streptomyces* sp	Slow and smooth	Cream	Brown	-
BPSEAC84	*Streptomyces* sp	Slow and rough	White	Orange	-
BPSEAC85	*Streptomyces* sp	Slow and rough	Cream white	Yellow	-
BPSEAC86	*Streptomyces* sp	Slow and rough	Cream	Brown	-
BPSEAC87	*Streptomyces* sp	Slow and smooth	White	Orange	-

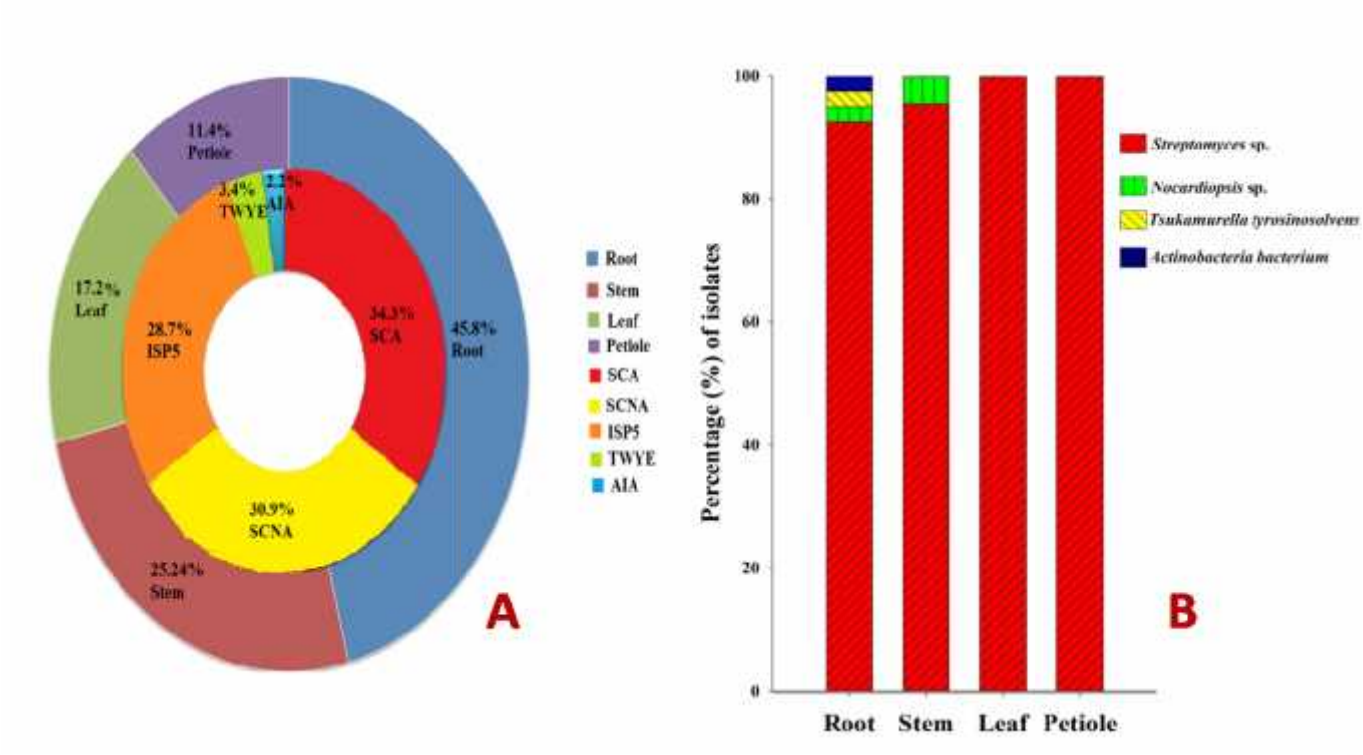

Fig 4.1: Distribution of endophytic actinomycetes isolates in different tissues and media used

Table 4.2: Distribution of endophytic actinomycetes isolated from the selected plants

Plant	**Number of isolates obtained**
Ageratum conyzoides	25
Mikania micrantha	30
Costus speciosus	15
Senecio scandens	10
Cassia fistula	7
Total	**87**

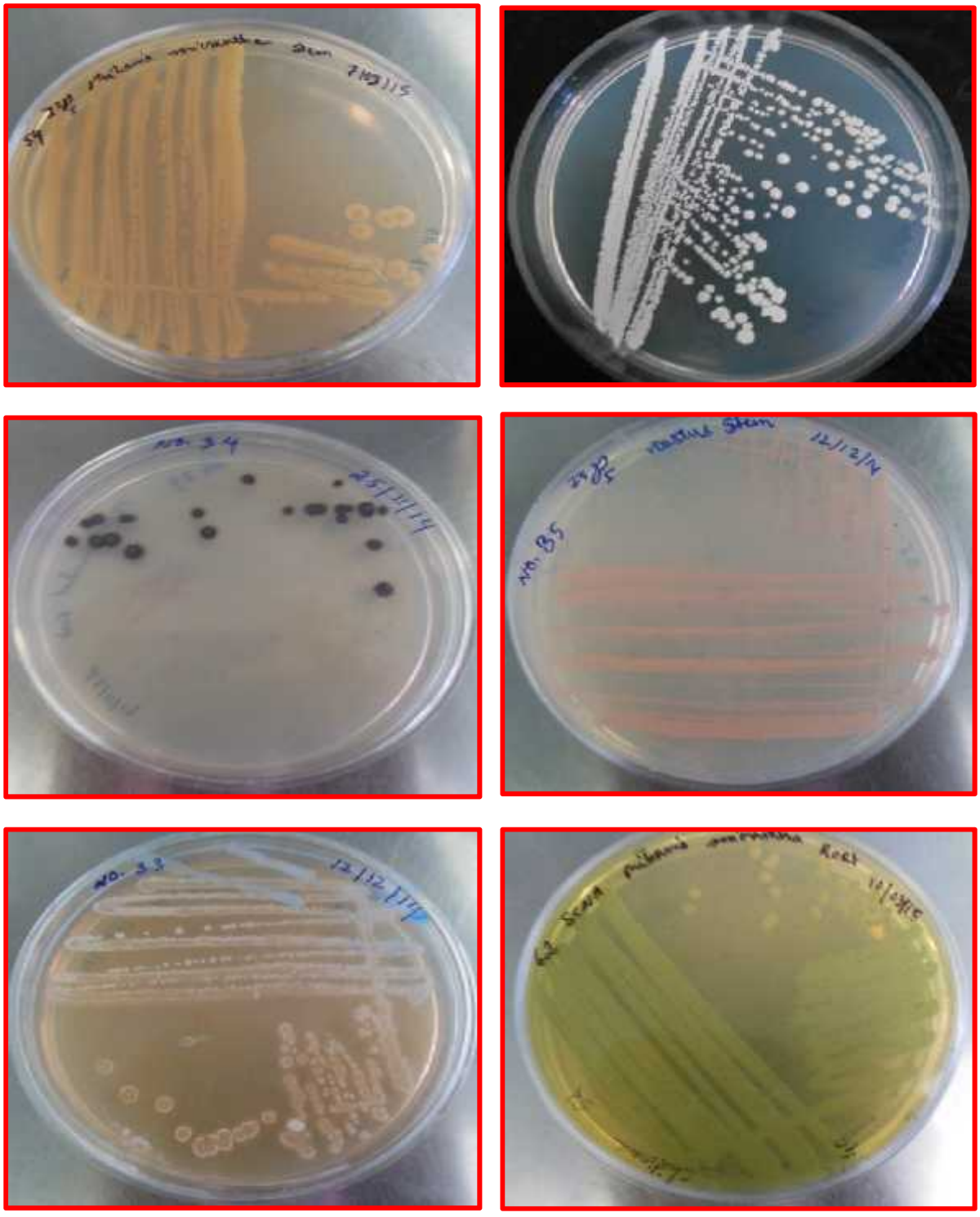

Fig4.3: Morphological characteristics of endophytic actinomycetes isolates

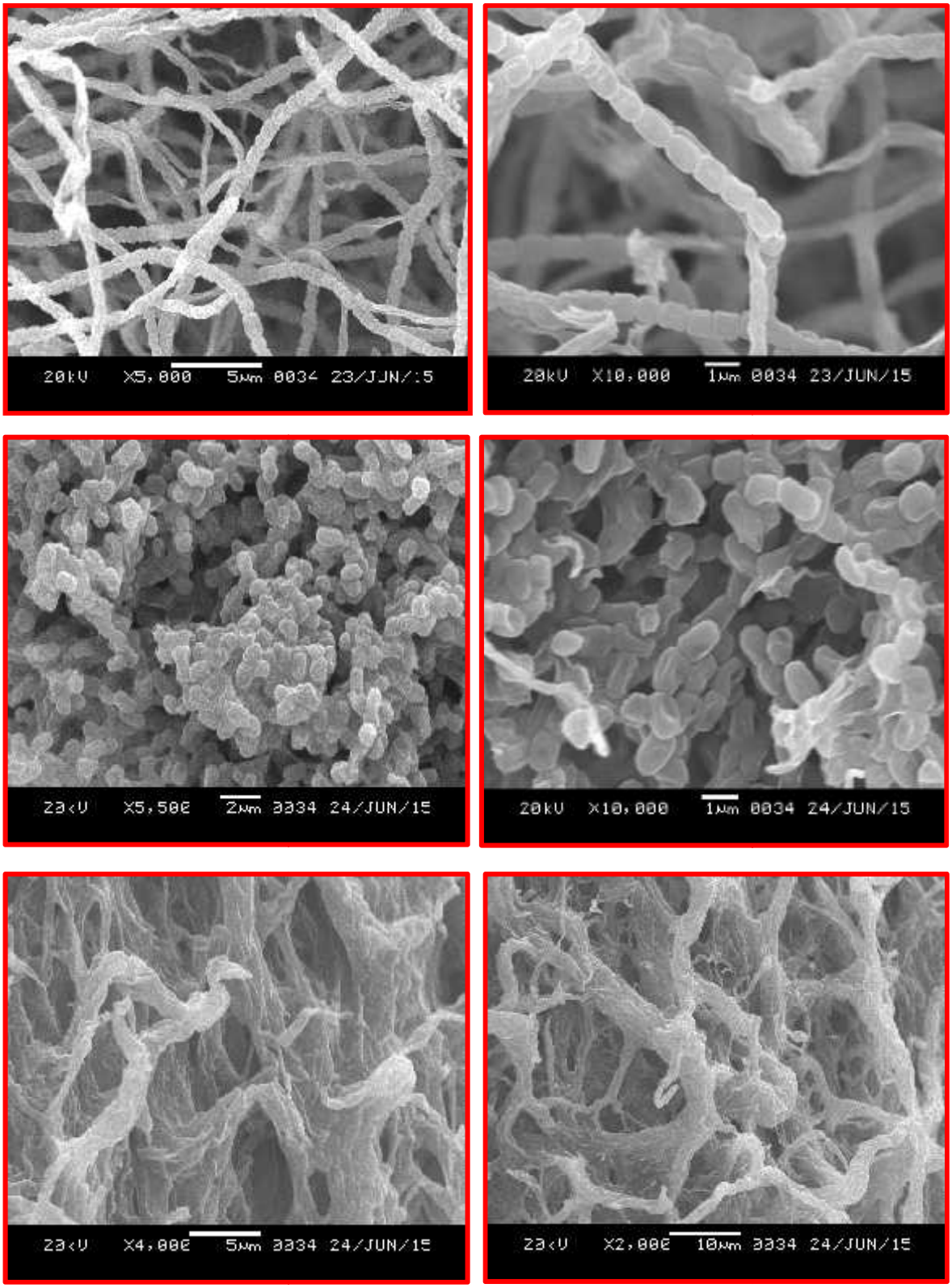

Fig4.4: Field Emission Gun-Scaning Electron Microscopy (FEG-SEM) characteristics of endophytic actinomycetes isolates

4.2. Evaluation of antifungal activity of endophytic actinomycetes against fungal pathogens

The antifungal activity of all the isolates was tested against four fungal phytopathogens viz, *Fusarium oxysporum f. ciceri, Fusarium proliferatum, Fusarium graminaerium and Fusarium udam.* Out of 87 endophytic actinomycetes, 46 (52.8%) isolates showed inhibitory activity against atleast two pathogens and among 46 isolates, two isolates BPSEAC40 *(Streptomyces sp.*) and BPSEAC11 (*Streptomyces olivaceus*) showed significant antifungal activity against all the four pathogens ranging from 36±0.2 to 56±0.1. The two isolates BPSEAC1 (*Streptomyces* sp.) and BPSEAC16 (*Streptomyces olivaceus*) showed inhibitory activity against three pathogens, ie. *Fusarium oxysporum ciceri, Fusarium proliferatum and Fusarium udam* ranging from 36±0.2 to 56±0.1 (Table 4.3 &Fig 4.5).

Table 4.3: Antagonistic potential of endophytic actinomycetes against tested four fungal pathogens

Isolate no.	Percentage of Inhibition (PI±SD)			
	Fusarium f. ciceri	*Fusarium proliferatum*	*Fusarium graminaerium*	*Fusarium udum*
BPSEAC1	52.0±0.05	36.0±0.20	-	56.0±0.10
BPSEAC2	44.8±0.07	-	-	38.0±0.10
BPSEAC3	39.6±0.02	-	-	42.0±0.05
BPSEAC4	37.6±0.02	-	-	48.0±0.05
BPSEAC5	58.0±0.07	-	-	44.0±0.05
BPSEAC6	56.0±0.10	-	-	44.0±0.05
BPSEAC7	48.0±0.10	-	-	40.0±0.10
BPSEAC8	40.8±0.07	-	-	41.0±0.02
BPSEAC9	38.0±0.10	-	-	37.0±0.02
BPSEAC10	44.8±0.02	-		46.0±0.05
BPSEAC11	46.8±0.05	44.0±0.05	40.0±0.10	48.0±0.05
BPSEAC12	49.6±0.05	-	-	35.0±0.10
BPSEAC13	37.6±0.11	-	-	37.0±0.02
BPSEAC14	38.8±0.05	-	-	44.0±0.05
BPSEAC15	42.4±0.05	-	-	40.0±0.05
BPSEAC16	41.6±0.02	40.0±0.10	-	37.0±0.02
BPSEAC17	38.0±0.05	-	-	42.0±0.05
BPSEAC18	42.8±0.05	-	-	41.0±0.02
BPSEAC19	43.6±0.02	-	-	40.8±0.07
BPSEAC20	52.0±0.05	-	-	44.8±0.05
BPSEAC21	48.0±0.10	-	-	36.0±0.10
BPSEAC22	35.6±0.10	-	-	52.0±0.05
BPSEAC23	57.6±0.11	-	-	52.0±0.10
BPSEAC24	52.0±0.05	-	-	40.0±0.10
BPSEAC25	37.6±0.02	-	-	35.0±0.10
BPSEAC26	46.8±0.05	-	-	44.0±0.02
BPSEAC27	41.6±0.02	-	-	40.0±0.10
BPSEAC28	35.6±0.10	-	-	45.6±0.10
BPSEAC29	37.6±0.11	-	-	42.4±0.10
BPSEAC30	38.0±0.10	-	-	36.4±0.06
BPSEAC31	39.6±0.02	-	-	44.8±0.05
BPSEAC32	44.8±0.07	-	-	42.0±0.10
BPSEAC33	40.8±0.07	-	-	49.6±0.02

BPSEAC34	57.6±0.11	-	-	37.6±0.10
BPSEAC35	49.6±0.05	-	-	41.2±0.02
BPSEAC36	42.4±0.05	-	-	38.8±0.06
BPSEAC37	35.6±0.10	-	-	38.8±0.30
BPSEAC38	35.6±0.10	-	-	45.2±0.03
BPSEAC39	37.6±0.11	-	-	41.6±0.10
BPSEAC40	56.0±0.10	40±0.10	54±0.05	41.2±0.05
BPSEAC41	44.8±0.07	-	-	44.0±0.04
BPSEAC42	44.8±0.07	-	-	46.0±0.08
BPSEAC43	39.6±0.02	-	-	42.0±0.08
BPSEAC44	43.6±0.02	-	-	38.0±0.10
BPSEAC45	46.8±0.05	-	-	42.4±0.10
BPSEAC46	35.6±0.10	-	-	37.6±0.10
BPSRAC47	46.8±0.05	-	-	48.0±0.05

Mean ± standard Deviation (SD) from triplicate samples.

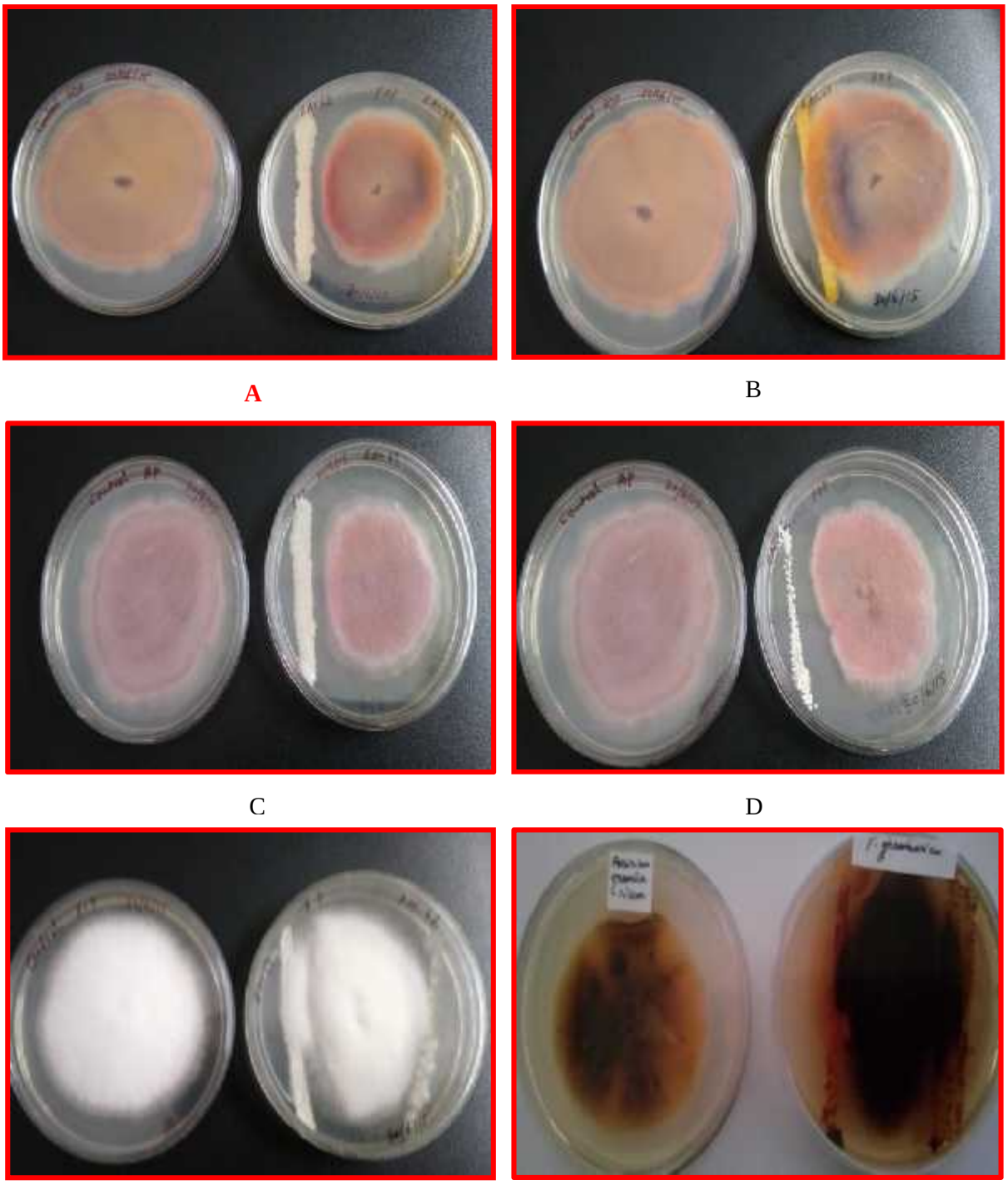

Fig 4.5: Antagonistic activity of endophytic actinomycetes against four fungal pathogens. A and B-Antifungal activity of BPSEAC40 against *Fusarium udum;* C and D- Antifungal activity BPSEAC40 against *Fusarium oxysporum f. ciceri*; E- Antifungal activity of BPSEAC40 against *Fusarium proliferatum; F*- Antifungal **activity of BPSEAC40 against *Fusarium graminaerium.***

4.3. Molecular characterization of endophytic actinomycetes

Potential antifungal isolates (n=46) based on antifungal screening were selected for molecular characterization. DNA was extracted by using Pure link Genomic DNA isolation Kit (In-vitrogen, Carlsbad, CA, USA) according to manufacturer's protocol (Fig 4.6).

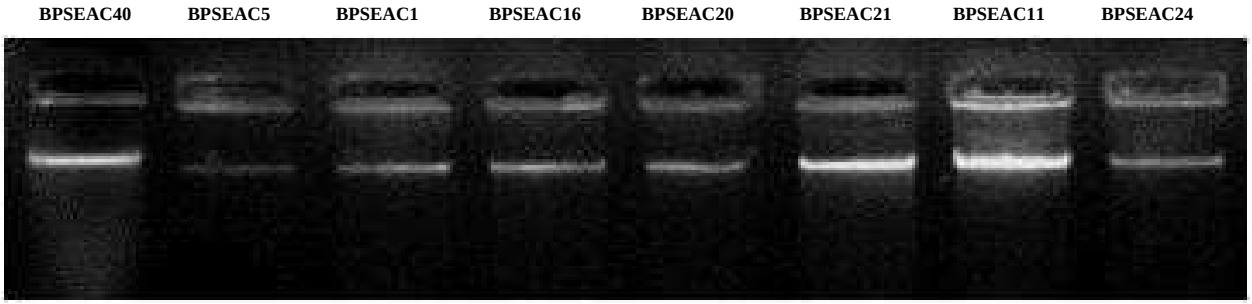

Fig 4.6: Isolated genomic DNA of endophytic actinomycetes of medicinal plants

4.4. PCR amplification of 16S rRNA geneof endophytic actinomycetes

Amplification of 16S rRNA gene is done by using universal primer (16SrRNA F5'-AGAGTTTGATCCTGGCTCA-3'and 16S rRNA R 5'-ACGGCTACCTTGTTACGACT-3') (Cui et al. 2001). A single 1500 bp amplicon was amplified as expected from all the selected isolates (Fig 4.7). Amplified PCR product was commercially sequenced and obtained sequences were analyzed by using basic bioinformatics tools (Blast) and sequences were submitted to NCBI GenBank. NCBI GenBank accession were obtained for all the isolates KU158241-KU158286 (46 in total) (Table 4.4).

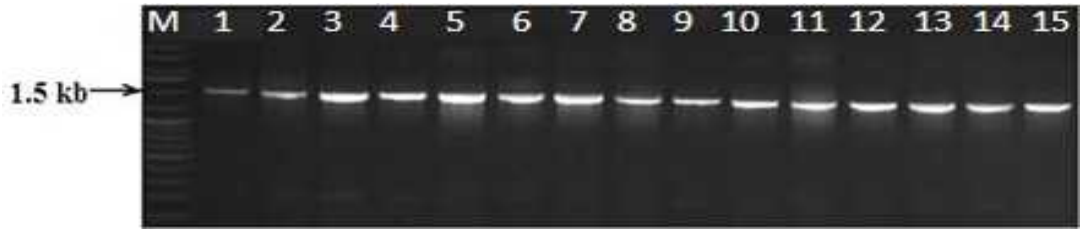

Fig. 4.7: Amplification of 16S rRNA gene: Molecular marker (M): low range DNA ruler plus Lane 1-14 different isolate

Table 4.4: Identification of antagonistic potential endophytic actinomycetes based on 16S r RNA Gene sequences.

Isolate no.	NCBI GeneBank accession no.	Closest species with accession number	Similarity	Identification
BPSEAC1	(KU158241)	*Streptomyces somaliensis* (KC98993)	99%	*Streptomyces* sp.
BPSEAC2	(KU158242)	*Streptomyces* sp.(KM220610)	99%	*Streptomyces* sp.
BPSEAC3	(KU158243)	*Nocardiopis* sp. *(KM886195)*	99%	*Nocardiopsis* sp.
BPSEAC4	(KU158244)	*Streptomyces* sp. (KP330251)	99%	*Streptomyces* sp.
BPSEAC5	(KU158245)	*Streptomyces thermocarboxydus* (KP128880)	99%	*Streptomyces thermocarboxydus*
BPSEAC6	(KU158246)	*Streptomyces* sp.(KF750593)	99%	*Streptomyces* sp.
BPSEAC7	(KU158247)	*Streptomyces* sp.(JN408756)	99%	*Streptomyces* sp.
BPSEAC8	(KU158248)	*Streptomyces* sp.(KJ143641)	100%	*Streptomyces* sp.
BPSEAC9	(KU158249)	*Streptomyces* sp.(KM253078)	100%	*Streptomyces* sp.
BPSEAC10	(KU158250)	*Streptomyces* sp.(JN969010)	99%	*Streptomyces* sp.
BPSEAC11	(KU158251)	*Streptomyces olivaceus* (KP128878)	99%	*Streptomyces olivaceus*
BPSEAC12	(KU158252)	*Streptomyces* sp.(DQ887329)	99%	*Streptomyces* sp.
BPSEAC13	(KU158253)	*Streptomyces* sp.(EU360158)	100%	*Streptomyces* sp.
BPSEAC14	(KU158254)	*Streptomyces* sp.(KM220610)	99%	*Streptomyces* sp.
BPSEAC15	(KU158255)	*Streptomyces* sp.(GU550569)	96%	*Streptomyces olivaceus*
BPSEAC16	(KU158256)	*Streptomyces* sp.(JQ422121)	96%	*Streptomyces olivaceus*
BPSEAC17	(KU158257)	*Streptomyces* sp.(KM253078)	99%	*Streptomyces* sp.
BPSEAC18	(KU158258)	*Streptomyces* sp.(JQ812094)	96%	*Streptomyces olivaceus*
BPSEAC19	(KU158259)	*Streptomyces* sp.(GU550569)	97%	*Streptomyces bikinensis*

BPSEAC20	(KU158260)	*Streptomyces* sp.(JN969010)	100%	*Streptomyces* sp.
BPSEAC21	(KU158261)	*Streptomyces* sp.(KP338793)	99%	*Streptomyces* sp.
BPSEAC22	(KU158262)	*Streptomyces olivaceus* (EU273545)	96%	*Streptomyces olivaceus*
BPSEAC23	(KU158263)	*Streptomyces* sp.(JQ812085)	95%	*Streptomyces olivaceus*
BPSEAC24	(KU158264)	*Streptomyces* sp.(KC462526)	97%	*Streptomyces prasinipilous*
BPSEAC25	(KU158265)	*Streptomyces* sp.(KM220610)	99%	*Streptomyces* sp.
BPSEAC27	(KU158266)	*Streptomyces violascens* (KP636799)	99%	*Streptomyces violascens*
BPSEAC28	(KU158267)	*Streptomyces albogriseoplanus.* (EU722759)	82%	*Streptomyces albogriseoplanus*
BPSEAC29	(KU158268)	*Streptomyces qriseoplanus* (HQ238386)	99%	*Streptomyces qriseoplanus*
BPSEAC30	(KU158269)	*Streptomyces violascens* (KM378575)	100%	*Streptomyces* sp.
BPSEAC31	(KU158270)	*Tsukamurella tyrosinosolvens* (AB480761)	99%	*Tsukamurella tyrosinosolvens*
BPSEAC32	(KU158271)	*Streptomyces* sp.(KT274752)	99%	*Streptomyces* sp.
BPSEAC33	(KU158272)	*Streptomyces* sp.(KR857308)	99%	*Streptomyces* sp.
BPSEAC34	(KU158273)	*Streptomyces collinus* (JX050226)	76%	*Streptomyces qriseoplanus*
BPSEAC35	(KU158274)	*Streptomyces olivaceus* (JN942120)	96%	*Streptomyces olivaceus*
BPSEAC36	(KU158275)	*Nocardiopsis* sp.(KF270095)	87%	*Nocardiopsis* sp.
BPSEAC37	(KU158276)	*Streptomyces violascens* (KT274752)	99%	*Streptomyces* sp.
BPSEAC38	(KU158277)	*Streptomyces daghestanicus* (KC747470)	99%	*Streptomyces* sp.
BPSEAC39	(KU158278)	*Streptomyces olivaceus* (KJ781985)	100%	*Streptomyces olivaceus*
BPSEAC40	(KU158279)	*Streptomyces anulatus* (KC814715)	100%	*Streptomyces* sp.

BPSEAC41	(KU158280)	*Streptomyces albidoflavus* (KP339504)	99%	*Streptomyces albidoflavus*
BPSEAC42	(KU158281)	*Streptomyces koyangensis* (KM678242)	100%	*Streptomyces specialis*
BPSEAC43	(KU158282)	*Actinobacteria bacterium* (KP053722)	99%	*Actinobacteria bacterium*
BPSEAC44	(KU158283)	*Streptomces violascens*(KP636799)	99%	*Streptomyces violascens*
BPSEAC45	(KU158284)	*Streptomyces* sp.(EU3601528)	99%	*Streptomyces* sp.
BPSEAC46	(KU158285)	*Streptomyces* sp.(KJ49330)	99%	*Streptomyces* sp.
BPSEAC47	(KU158286)	*Streptomyces violascens* (KP636799)	99%	*Streptomyces* sp.

4.5. 16S rRNA Sequence alignment and phylogenetic analysis of endophytic actinomycetes

To investigate the relationships among the more promising endophytic actinomycetes isolate, 16S rRNA gene sequences were aligned along with the sequences of type strains retrieved from DDBJ/EMBL/NCBI GenBank databases. The results showed that the isolates were classified into four families and four genera. Most of the isolates grouped into *Streptomycetaceae* (91.3%), followed by *Nocardiopsaceae* (4.34%), *Tsukamurellaceae* (2.17% each) and *Actinomycetaceae* (2.17% each). Analysis of the 16S rRNA gene sequence by BlastN with 98-100% similarity confirmed that 42 isolates could be members of genus *Streptomyces*. The sequences of the 2 isolates (BPSEAC3 and BPSEAC36) showed 99-100% identity to the sequences retrieved from genus *Nocardiopsis prasina* DSM438and isolates BPSEAC31 and BPSEAC43 showed high identity (99% each) to the genus *Tsukamurella tyrosinosolvens* and *Actinomycete*, respectively. The phylogenetic tree was constructed based on Neighbor-joining method with Kimura 2-parameter model (R=1.04) according to lowest BIC values using Mega 5.05 and the estimated Transition/Transversion bias (R) is 1.07. The topology of the phylogenetic tree generated which showed that all *Streptomyces* forms a major clade I, along with the type strains retrieved from databases with the exception to *Actinomycete*, which also falls in the same clade under a bootstrap support value of 64%. Most of the putative species in the genera *Nocardiopsis* and *Tsukamurella*, clustering to form another clade II under bootstrap value of 83% (Fig 4.8).

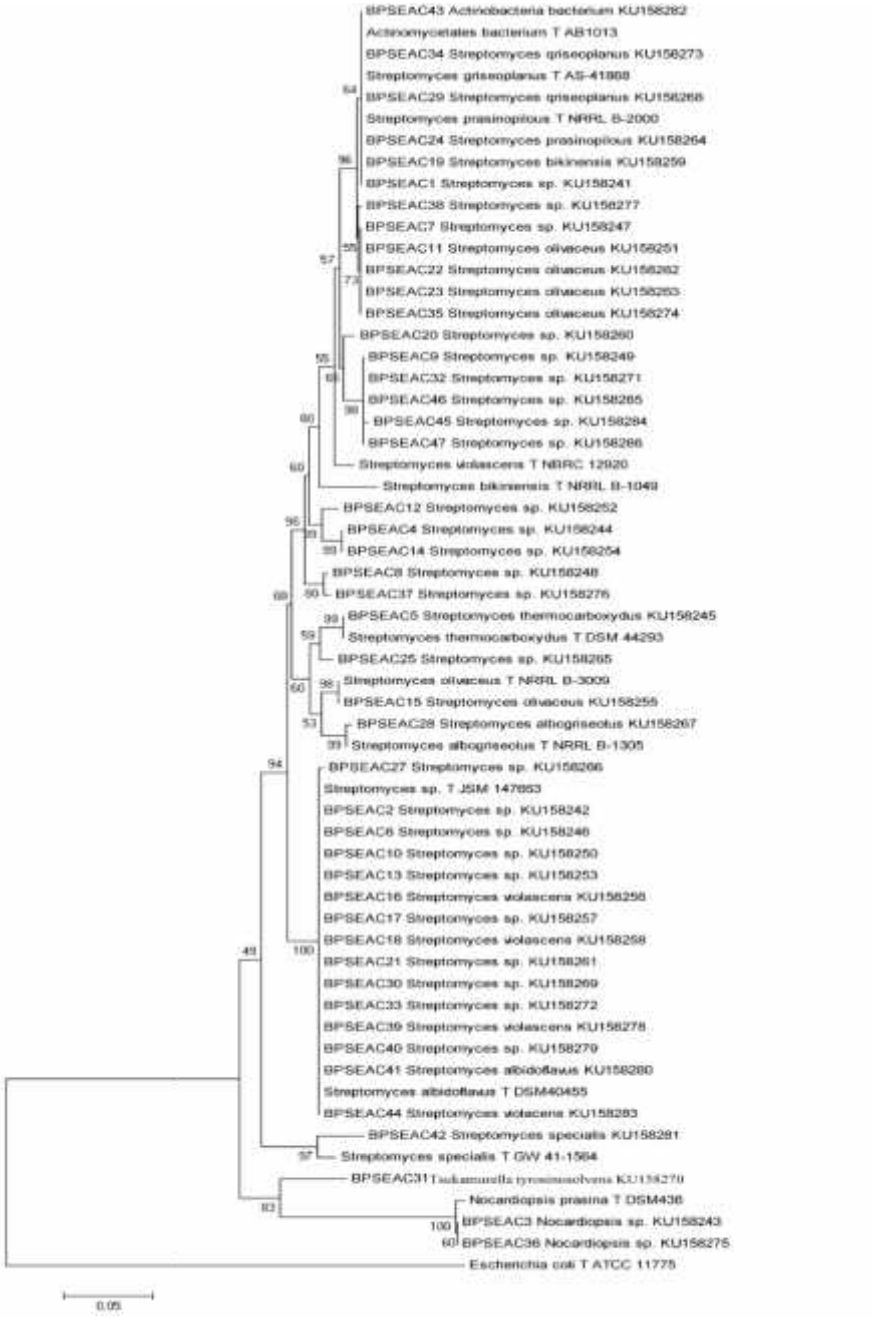

Fig 4.8: Neighbor-joining phylogenetic tree based on 16SrRNA gene of endophytic actinomycetes

4.6. BOX-PCR Fingerprinting of endophytic actinomycetes

Further, all isolates were genotypically compared by using BOX-PCR fingerprints and which consequently divided the isolatesinto five clusters (A-E) (Fig 4.10). The BOX-PCR fingerprinting pattern of all the endophytic bacteria revealed 3 to 12 fragments ranging in size from approx. <100 bp to 2.0 kb. Less visible fragments above 2kb was also found in some isolates (Fig 4.9). The cluster A contains 19 isolates consisting different genera belongs to

Streptomyces, Nocardiopsis and Actinomycete. Cluster B contains 19 isolates whereas cluster C comprised consists of 5 isolates. Cluster B and C consists of only genus *Streptomyces*. Cluster D was containing one isolates; interestingly belongs to genus *Streptomyce* group and fifth cluster E contains only one isolates belongs to *Tsukamurella* group (Fig4.10). The majority of the isolates analyzed showed different BOX fingerprinting patterns, which further confirms the high discriminative power of the BOX-PCR fingerprinting technique and reports high diversity of endophytic actinomycetes.

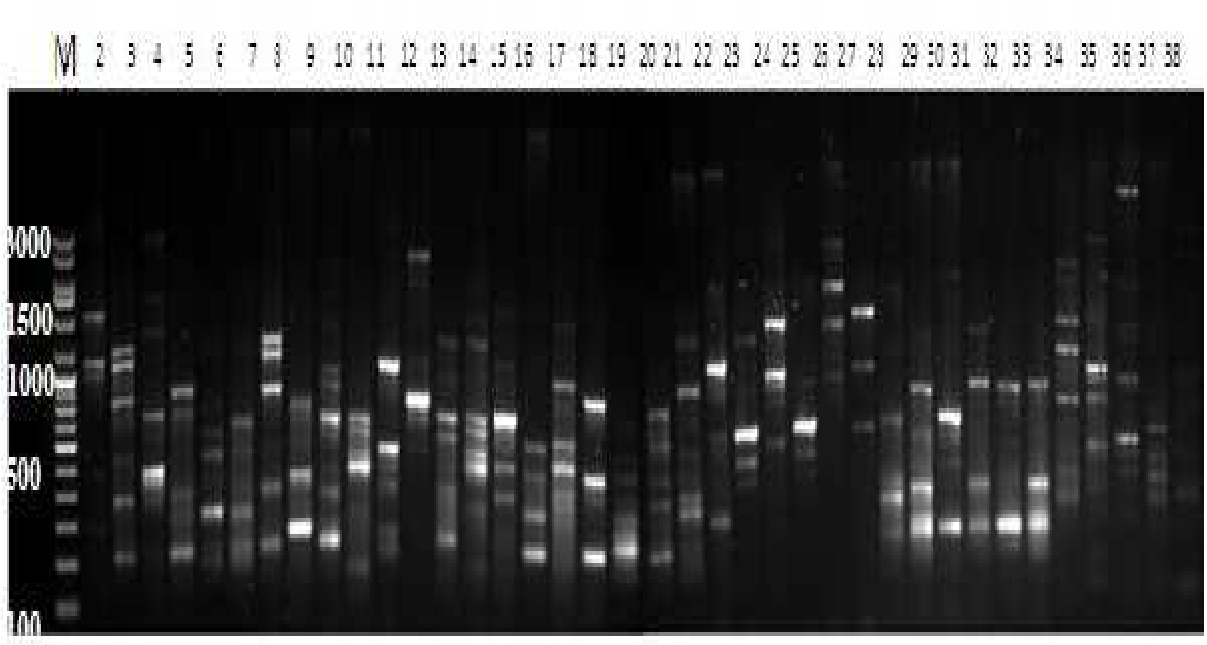

Fig 4.9: BOX-PCR amplification for endophytic actinomycetes, 100bp-3.0kb (M)-molecular markers; numerical numbers represent different isolates

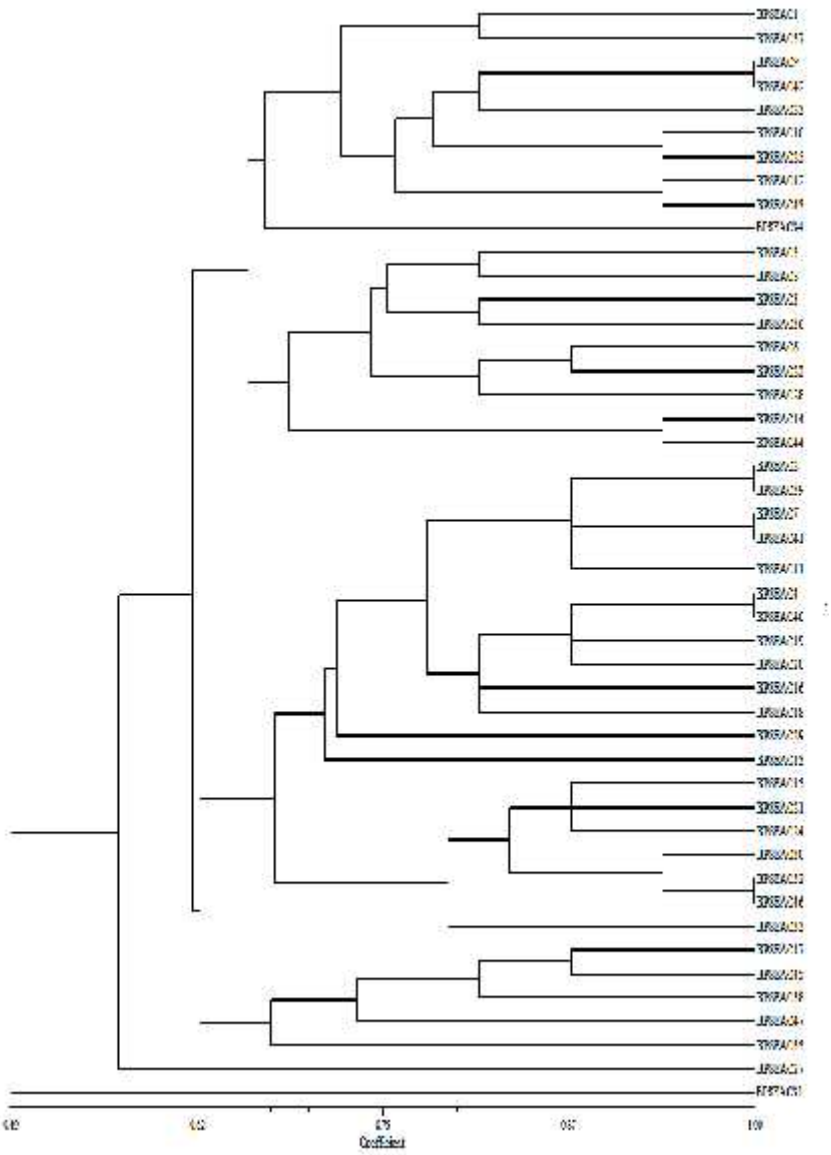

Fig 4.10: Dendrogram generated from BOX-PCR fingerprint patterns of the endophytic actinomycetes.

4.7. ERIC-PCR Fingerprintingof endophytic actinomycetes

All the antagonistic endophytic actinomycetes generated specific pattern by using ERIC-PCR and genetic diversity of isolates was not significant from both the locations. The fingerprinting pattern yielded discriminatory patterns with 3 to 12 fragments ranging in size from approx. <100 bp to 3.0 kb which shows this technique was useful to allow differentiation among the isolates (Fig 4.11). Dendrogram generated by ERIC-PCR divided the isolates into five clusters (A-E) Cluster A containing 15 isolates; interestingly all belongs to genus *Streptomyces*. Cluster B was the largest cluster composed of 19 isolates all belongs to genus *Streptomyces* and genus *Actinomycete* falls in same Cluster. This is similarly found

in 16S rRNA phylogenetic tree and BOX-PCR result. Third cluster C contains 4 isolates comprising different genera belongs to *Tsukamurell*a and *Streptomyces*. Fourth cluster D consists of six isolates again all belong to genus *Streptomyces*. The last cluster E consists of two isolates composed of *Nocardiopsis* group(Fig4.12).

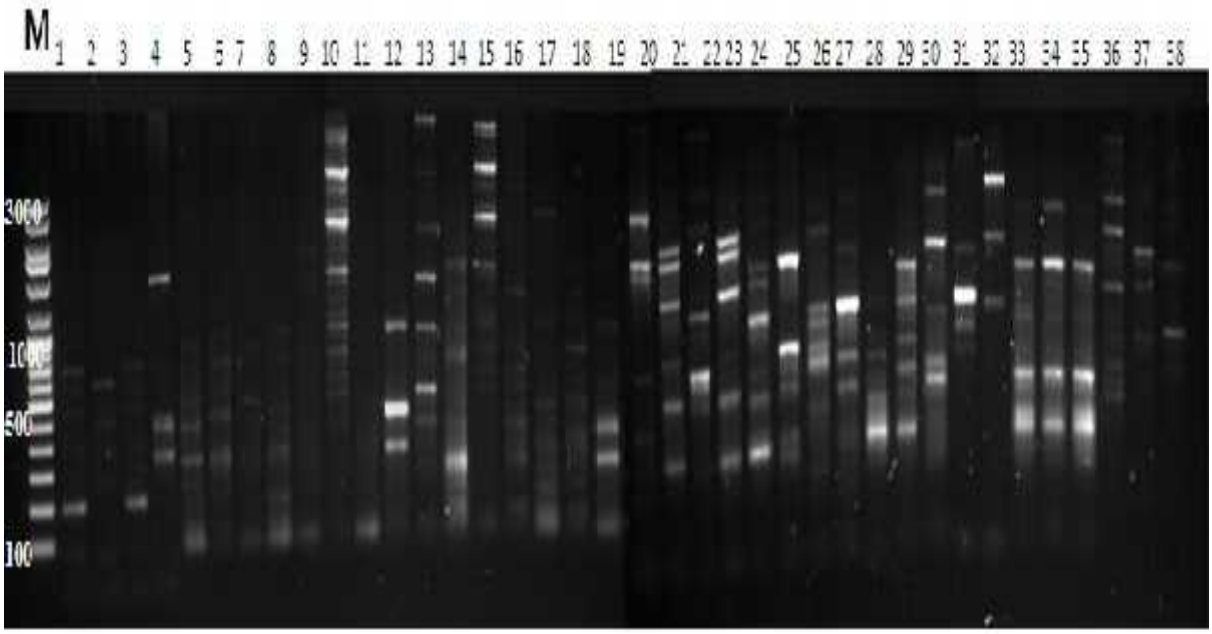

Fig4.11: ERIC-PCR amplification for endophytic actinomycetes, 100bp-3.0kb (M) molecular markers; numerical numbers represent different isolates.

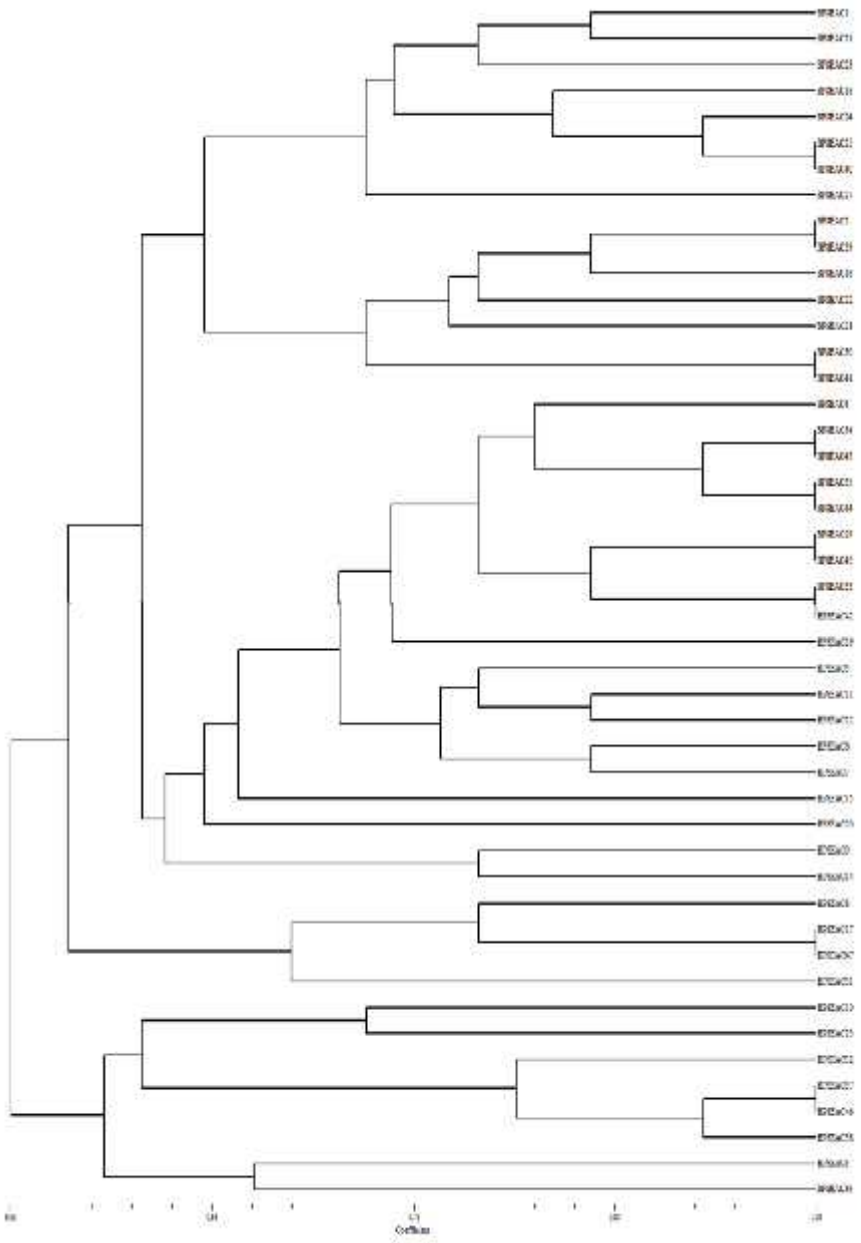

Fig4.12: Dendrogram generated from ERIC-PCR fingerprint patterns of the endophytic actinomycetes isolates

4.8. Plant growth promoting activities of antagonistic endophytic actinomycetes

4.8.1. Phosphate solubilization of endophytic actinomycetes

Among the 46 endophytic actinomycetes, 16 (34.7%) isolates were able to solubilize inorganic phosphate and were identified as potential phosphate solubilizing isolates based on a clear halo zone around the colony on Pikovskaya`s medium. The zone of phosphate solubilization varied from 1.3 to 2 cm and the highest phosphate solubilization were observed in BPSEAC43 and BPSEAC35 (identified as) *Actinobacteria bacterium* and *Streptomyces* sp.

(2±0.1) followed by BPSEAC38 and BPSEAC11 (identified as) *Streptomyces* species and *Streptomyces olivaceus*(1.96 ±0.35) and BPSEAC31 (identified as) *Tsukamurella* species (1.93±0.11)(Table 4.5) (Fig 4.13).

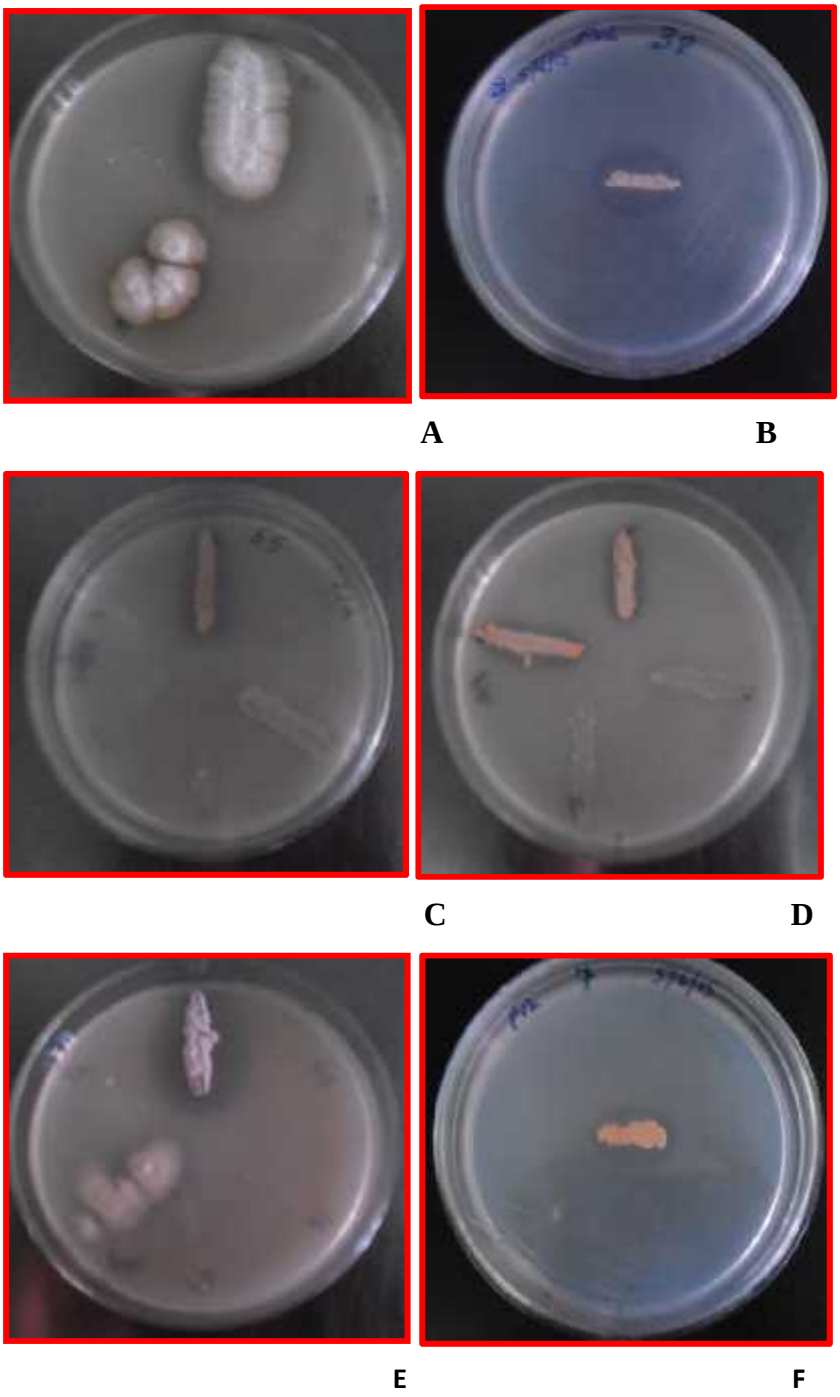

Fig4.13: Phosphate solubilization of endophytic actinomycetes. A- BPSEAC1, B- BPSEAC11, C- BPSEAC21, D- BPSEAC8, E- BPSEAC40 and F- BPSEAC20.

4.8.2. Production of indole-3-acetic acid of endophytic actinomycetes

All total46 (100%) isolates of endophytic actinomycetes were observed positive for IAA production. Quantitative analysis showed the amount of IAA produced ranging from 13.0µM/ml and 101.4 µM/ml. The highest amount of IAA production was produced by *Streptomyces olivaceus*(101.4±0.23)(BPSEAC11) followed by *Tsukamurella* species (101.0±0.28) (BPSEAC31) and *Streptomyces* sp. (BPSEAC6) (76.8±0.46) (Table 4.5).

4.8.3. Production of ammonia of endophytic actinomycetes

All 46 (100%) isolates of endophytic actinomycetes were positive for the production of ammonia. Quantitative estimation showed amount of ammonia production ranging from 19.0µM/ml and 145.3µM/ml. Isolates*Streptomyces olivaceus* (145.3±0.17)and *Tsukamurella species*(145.3±0.17)(BPSEAC11 and BPSEAC31) and showed maximum amount of productionn followed by *Streptomyces* sp. (BPSEAC37)and*Streptomyces* species (BPSEAC45) (131.3±0.17)(Table 4.5)**.**

4.8.4. Production of cellulase, amylase and catalase of endophytic actinomycetes

Screening of cellulase production showed 28(60.8%) isolates were positive among all 46 isolates of endophytic actinomycetes. Quantification showed cellulase activity ranging from 0.1052 U/ml and 0.4605 U/ml. Maximum cellulase activity was found in *Tsukamurella*sp. (0.4605±0.0008) (BPSEAC31)followed by*Streptomces species* (BPSEAC20) (0.4581±0.0005) and*Streptomycesolivaceus* (0.4509±0.0105) (BPSEAC11)(Table 4.5) (Fig4.14).

Out of 46 isolates, 40(86.9%) isolates of endophytic actinomycetes were observed production of amylase, forming clear zone around the colonies. Quantification estimation resulted amount of amylase varied from 0.1132 U/ml and 1.0449 U/ml. Isolate *Streptomyces bikinensis* (1.0813±0.0002) (BPSEAC19)observed maximum among 45 isolatesfollowed by*Streptomycesviolascens* (BPSEAC31)and *Tsukamurell*a species (1.0449±0.0050) (BPSEAC31) and *Streptomyces thermocarboxydus* (0.9467±0.0058) (Table 4.5) (Fig 4.14).

Among 46 isolates, 14 (30.4%) isolates were positive for catalase production as they formed effervescence (Table 4.5).

Fig 4.14: Screening of cellulase and amylase production of endophytic actinomycetes

Table 4.5: Plant growth promoting activitiesof antagonistic endophytic actinomycetes

Isolate no.	Phosphate	IAA µM/ml at 530nm	Ammonia µM/ml at 480nm	Cellulase (U/ml)	Amylase (U/ml)	Catalase Formed of effervescence (+/ -)
BPSEAC1	1.46±0.05	46.2±0.11	59.3±0.55	-	0.3822±0.0002	-
BPSEAC2	-	13.0±0.05	118.1±0.28	0.1492±0.0009	0.3823±0.0097	-
BPSEAC3	-	40.5±0.28	45.3±0.18	0.3147±0.0006	0.3406±0.0062	-
BPSEAC4	-	27.9±0.51	39.4±0.51	0.1051±0.0001	0.3849±0.0147	-
BPSEAC5	1.4±0.05	21.5±0.18	49.0±0.05	-	0.9467±0.0058	-
BPSEAC6	-	76.8±0.46	112.0±0.11	-	0.1702±0.0002	-
BPSEAC7	-	16.3±0.17	122.2±0.40	-	0.3236±0.0017	-
BPSEAC8	1.83±0.28	39.2±0.05	19.0±0.05	-	0.7183±0.0003	+
BPSEAC9	-	18.5±0.28	22.6±0.28	-	-	-
BPSEAC10	-	54.5±0.28	118.3±0.51	0.3101±0.0087	0.5010±0.0008	-
BPSEAC11	1.96±0.15	101.4±0.23	145.3±0.17	0.4509±0.0105	1.0449±0.0050	+
BPSEAC12	-	53.1±0.05	55.6±0.34	0.1349±0.0008	0.8536±0.0689	-
BPSEAC13	-	40.7±0.40	40.1±0.23	0.1381±0.0001	0.3738±0.0005	-
BPSEAC14	-	50.0±0.05	45.2±0.46	0.1380±0.0002	0.9493±0.0034	-
BPSEAC15	-	63.0±0.05	119.2±0.28	0.1028±0.0026	0.1229±0.0056	-
BPSEAC16	1.46±0.05	17.6±0.34	44.1±0.17	-	0.1591±0.0002	+
BPSEAC17	-	18.5±0.28	33.6±0.34	-	0.1133±0.0001	+
BPSEAC18	-	28.3±0.17	23.6±0.34	0.1423±0.0026	0.8137±0.0002	+
BPSEAC19	-	19.1±0.05	42.1±0.28	0.2629±0.0009	1.0813±0.0002	-
BPSEAC20	1.4±0.05	33.6±0.34	33.8±0.34	0.4581±0.0005	-	+
BPSEAC21	1.4±0.05	22.0±0.05	70.1±0.23	0.2378±0.0007	0.6761±0.0140	+
BPSEAC22	-	23.4±0.05	71.1±0.23	-	0.3784±0.0120	-
BPSEAC23	1.8±0.28	41.4±0.23	109.5±0.28	-	0.7827±0.0514	-
BPSEAC24	-	17.4±0.34	40.6±0.23	0.2378±0.0007	0.5213±0.0771	-
BPSEAC25	-	64.2±0.28	89.1±0.17	-	0.2399±0.0270	-

BPSEAC27	-	46.5±0.17	136.8±0.46	0.2626±0.0013	0.9023±0.0058	-
BPSEAC28	-	35.8±0.28	92.2±0.11	-	0.5450±0.0363	-
BPSEAC29	-	50.3±0.05	43.2±0.40	-	0.3962±0.0049	-
BPSEAC30	-	33.7±0.28	110.2±0.11	0.2231±0.0098	-	+
BPSEAC31	1.93±0.11	101.0±0.28	145.3±0.17	0.4605±0.0008	1.3615±0.0016	-
BPSEAC32	-	21.0±0.05	32.1±0.28	-	0.2252±0.0218	+
BPSEAC33	-	16.3±0.34	73.5±0.23	0.1467±0.0058	0.3719±0.0015	-
BPSEAC34	-	23.4±0.05	73.2±0.23	0.1623±0.0024	0.6279±0.0245	-
BPSEAC35	2±0.1	39.2±0.20	28.1±0.17	0.3688±0.0058	0.3620±0.0220	-
BPSEAC36	1.4±0.2	26.2±0.17	32.1±0.34	-	0.6398±0.0199	-
BPSEAC37	--	35.0±0.28	144.0±0.17	0.1718±0.0006	-	-
BPSEAC38	1.96±0.35	63.6±0.28	123.2±0.34	0.1511±0.0002	0.7038±0.0057	-
BPSEAC39	-	50.2±0.05	118.4±0.28	0.1423±0.0003	0.6524±0.0457	-
BPSEAC40	-	46.5±0.46	40.9±0.2	-	0.5014±0.0002	+
BPSEAC41	1.4±0.2	28.7±0.40	64.1±0.28	0.2702±0.0003	0.5616±0.0072	-
BPSEAC42	-	17.6±0.34	36.7±0.40	-	0.3241±0.0027	+
BPSEAC43	2±0.1	63.6±0.28	123.6±0.34	-	0.8935±0.0005	+
BPSEAC44	-	34.3±0.34	38.6±0.34	-	-	-
BPSEAC45	1.5±0.1	20.2±0.05	131.3±0.17	0.1371±0.0002	0.3636±0.0205	+
BPSEAC46	1.4±0.2	50.0±0.05	31.3±0.17	-	0.9513±0.0022	+

Mean ± standard error from triplicate samples.

CHAPTER 5

DISCUSSION

Endophytes are widely distributed in nature and producer of diverse prospective natural bioactive compounds (Zhao *et al.,* 2012). Previous studies have reported the isolation of endophytic actinomycetes from a variety of plants ranging from crop plants, such as wheat, rice, potato, carrots, tomato, and citrus (Tian *et al.,* 2007), different woody tree species (Zhao *et al*., 2010a,b,c), ferns and club mosses (Janso and Carter, 2010). Many researchers have been reported that endophytic actinomycetes associated with medicinal plants are valuable source of natural products with potential bioactivities (Inderiati and Christopher, 2008). Endophytes of medicinal plants take part in metabolic pathways and generate analogous novel bioactive compounds (Zhao *et al*., 2011). However, there are not much studies regarding the microbiological studies on endophytic actinomycetes of medicinal plants of Mizoram. This importance encouraged us to explore and investigate the endophytic actinomycetes associated with ethnomedicinal plants of Mizoram to understand the role of endophytic actinomycetes in plants and their production of potential compounds with desired bioactivities as antagonistic and plant growth promoting activity. In this study, we have explored endophytic actinomycetes associated with traditional medicinal plants of Mizoram, and a certain group of endophytic actinomycetes were found which displayed specific activity of antagonistic and plant growth promoting were observed.

In this study, we obtained 87 strains of endophytic actinomycetes from five medicinal plants of Mizoram. Majority effective isolates were recorded in Starch Casein Agar (SCA) media followed by Starch Casien Nitrate Agar (SCNA) and International Streptomyces Project 5 (ISP5) media. Similar result were also found who has been reported that most frequently isolated endophytic actinomycetes from the medium ISP5 (Inderiati and Christopher, 2008), SCNA (Passari *et al*., 2015) and SCA (Küster and Williams, 1964).Among total isolates *Streptomyces* was the dominant genus (n=81, 93.1% of isolates), which was consistent with other reports from different hosts (Tanvir *et al*., 2014). Besides *Streptomyces* sp., other genera like *Tsukamurella species*, *Nocardiopsis species*, *Actinobacteria species* was also obtained which was consistent with the resultsisolated from *Maytenus austroyunnanesis* (Qin *et al.,* 2012), *Nocardia* sp. explored from *Artemisia judaica*from *Mirabilis jalapa* (Golinska *et al.,* 2015) and*Actinomycete* sp. from *Mirabilis jalapa* (Passari *et al*., 2015, Kumar *et al*., 2011) were also reported as rare endophytic actinomycetes from medicinal plants. The

extension of diversity of endophytic actinobacterial communities may vary between different sample collection regions and different plant species (Qin *et al.,* 2011). The genus *Streptomyces* was an excellent producers of bioactive metaboilites as they serve as sources of novel bioactive products (Ryan *et al*., 2008). Endophytic actinomycetes can be isolated from all the available tissues in plant, however, present study resulted majorityorganisms were recorded from roots(n=40, 45.8%)followed by stem(n=22, 25.4%), leaves (n=15, 17.2%) and petioles (n=10, 11.4%) these findings indicated that endophytic actinomycetes are most dominant in root tissues. Our results are consistent with the reported by Zhao *et al.,* (2011) obtained 560 endophytes from 26 species of healthy medicinal plants where more number of isolates were recovered from roots (58.2%), followed by stems (27.8%) and leaves (14%). These findings revealed that roots represent favourable habitat for endophytic actinomycetes. This may be because of actinomycetes are natural soil dweller and prevalence in rhizospheric soil and enter intact plant tissue by invagination of root hair cell wall, by penetration of the junction between root hair and adjacent epidermal cells (Passari *et al.,* 2015).

All the isolates were screened for their potential antagonistic activity against four fungal phytopathogens. Among them 46 isolates could produce antagonistic activity against *Fusarium oxysporum* f. *ciceri* and *Fusarium udam*. Most of them were belonged to genus *Streptomyces.* Among them,two isolates BPSEAC40 *(Streptomyces sp.*) and BPSEAC11 (*Streptomyces olivaceus*) showed significant antifungal activity against all the four pathogens. The two isolates BPSEAC1 (*Streptomyces* sp.) and BPSEAC16 (*Streptomyces olivaceus*) showed inhibitory activity against three pathogens, ie. *Fusarium oxysporum ciceri, Fusarium proliferatum and Fusarium udam.*Similarly, Passari *et al.,* 2015 tested endophytic actinomycetes against *Fusarium oxysporum* f. *ciceri* and *Fusarium graminaerium*. His results showed significant inhibitory activity against *Fusarium graminaerium*. Li *et al*., (2008) suggested that it may be due to the fact that endophytic *Streptomyces* isolated from medicinal plants were expected to produce a wide variety of bioactive compounds. Many endophytic actinobacteria, especially those from medicinal plants possess the ability of inhibiting a wide variety of harmful microorganisms like pathogenic bacteria, fungi and viruses (Qin *et al*., 2011).

Out of total 46 isolates,16 (34.7%)were observed to solubilize phosphate. The maximum amount of phosphate solubilization was shown by BPSEAC43 and BPSEAC35 (identified as) *Actinobacteria bacterium* and *Streptomyces* sp. (2±0.1) followed by BPSEAC38 and

BPSEAC11 (identified as) *Streptomyces* species and *Streptomyces olivaceus*(1.96 ±0.35) and BPSEAC31 (identified as) *Tsukamurella* species (1.93±0.11)(Table.6). Similarly, 22 endophytic actinomycetes isolated from medicinal plants, 14 (63.6%) were able to solubilize inorganic phosphate and the highest solubilization was detected in *Streptomyces* species. It was reported that the microbial phosphate solubilization may be either due to the acidification of external medium or the production of chelating substances that increases the solubilization of mineral phosphate (Welch *et al.,* 2002). Biological phosphate solubilisation as an alternative to natural phosphate utilization plays an important role in efficient nutrient uptake (Kaur *et al.,* 2013). Phosphate solubilizing microorganisms not only supply plants with phosphorus but they are also accelerates the accessibility of other trace elements (Mittal *et al.,* 2008). Hence, endophytic actinomycetes with phosphate solubilization activity play role in enhancing plant growth and development.

All the 46 isolates showed production of IAA and 41 (89.1%) of these belonged to *Streptomyces* sp. The range of IAA production was 13.0µM/ml and 101.4 µM/ml. The highest amount of IAA production was produced by *Streptomyces olivaceus* (BPSEAC11) (Table 6). Previous studied by Dochhil *et al.,* 2013 demonstrated that the two *Streptomyces* sp. isolated from *Centella asiatica* were evaluated indole acetic acid (IAA) and found plant growth enhancement and higher seed germination percentage in much higher concentration as 71 g/ml and 197 g/ml. Similarly, Gangwar *et al*., (2014) studied showed that mostly *Streptomyces* sp. were found to be capable of producing IAA within the range of 9.0-38.8 µg/ml. IAA is a natural auxin which is a product of L-Tryptophan metabolism in microorganisms. It can be due to the high levels of Tryptophan present in the tissues of medicinal plants which enhanced IAA biosysnthesis.Hence, endophytic actinomycetes produce IAA may play role in plant health and their growth.It plays important roles in a number of plant activities, including: development of the embryo, leaf formation, phototropism, gravitropism, apical dominance, fruit development, abscission, root initiation and development.

In this study, all 46 endophytic actinomycetes was observed production of ammonia. Ammonia producing actinomycetes falls in the ranges from 19.0 to 145.0µM/ml. Similarly, according to Kaur *et al.,* (2013) ammonia was detected for endophytic actinomycetes and confirmed 13 isolates positive out of 15 endophytic actinomycetes. Marques *et al*., (2010) suggested that bacteria produced ammonia and supply nitrogen to the host plant. Endophytes

produced ammonia developed plant root and shoot elongation, as a result increased plant biomass.

Out of 46 tested isolates to detect the production of cellulase, 28(60.8%) isolates were able to produced cellulase substances. On the other hand, 40(86.9%) isolates showed amylase activity. These findings corroborates the result obtained by the previous studies Ramesh and Mathivanan, (2009) have been proven that some actinomycetes produced hydrolytic enzymes such as cellulase, amylase, chitinase and protease. Lima *et al*., (1998) reported that microorganisms produced cellulases play an important role in controlling of growth of plant pathogens like Phytopthora and Pythium.

Out of 46 isolates,14 (30.4%) was produced catalase. Our results is similar with the previous studied by Sousa *et al*., (2008) observed that all *Streptomyces* isolates produced catalase, amylase, lipase. Since endophytic actinomycetes has been considered as potential bioactive compounds. The presence of endophytic actinomycetes produced catalase is an important source as biological control agents and consequently promote growth of plant.

CONCLUSION

In the present study, from various tissues samples of five medicinal plants 87 number of endophytic actinomycetes were obtained. Based on *in vitro*antagonisticpotential activity we selected 46 isolates for identification (molecular characterization) and plant growth promoting activities. Endophytic actinomycetes associated with medicinal plants is been reported as apotential source for the discovery of bioactive compounds. Hence, in the present study, the traditionally use medicinal plants were selected for the exploration of endophytic actinomycetes. In total, 87 isolates were confirmed actinomycetes based on their morphological and microscopic characteristics. All the isolates were screened for their in-vitro antagonised potential against four major fungal pathogens and the isolates which showed antagonistic activity against more than one pathogen were further selected for molecular characteristics and we screened for their plant growth promoting traits. Out of 87 isolates, 46 isolates showed antagonistic potential against more than one pathogen, were screened for their plant growth promoting abilities. Among the 46 endophytic actinomycetes, 16 (34.7%) isolates were able to solubilize inorganic phosphate.The zone of phosphate solubilization varied from 1.3 to 2 cm.All total 46 (100%) isolates of endophytic actinomycetes were observed positive for IAA production. Quantitative analysis showed the amount of IAA produced ranging from 13.0µM/ml and 101.4 µM/ml. The highest amount of IAA production was produced by *Streptomyces olivaceus*(101.4±0.23)(BPSEAC11) followed by *Tsukamurella* species (101.0±0.28) (BPSEAC31) and *Streptomyces* sp. (BPSEAC6) (76.8±0.46). All 46 (100%) isolates of endophytic actinomycetes were positive for the production of ammonia. Quantitative estimation showed amount of ammonia production ranging from 19.0µM/ml and 145.3µM/m. Isolates*Streptomyces olivaceus* (145.3±0.17)and *Tsukamurella species* (145.3±0.17) (BPSEAC11 and BPSEAC31) and showed maximum amount of productionn followed by *Streptomyces* sp. (BPSEAC37)and*Streptomyces* species (BPSEAC45) (131.3±0.17).Screening of cellulase production showed 28 (60.8%) isolates were positive among all 46 isolates of endophytic actinomycetes. Quantification showed cellulase activity ranging from 0.1052 U/ml and 0.4605 U/ml. Maximum cellulase activity was found in *Tsukamurella*sp. (0.4605±0.0008) (BPSEAC31)followed by *Streptomces species* (BPSEAC20) (0.4581±0.0005) and *Streptomycesolivaceus* (0.4509±0.0105) (BPSEAC11). Out of 46 isolates, 40(86.9%) isolates of endophytic actinomycetes were observed production of amylase, forming clear zone around the colonies. Quantification estimation resulted amount of amylase varied from 0.1132 U/ml and 1.0449 U/ml. Isolate *Streptomyces bikinensis* (1.0813±0.0002) (BPSEAC19)observed maximum among 45

isolatesfollowed by*Streptomycesviolascens* (BPSEAC31)and *Tsukamurell*a species (1.0449±0.0050) (BPSEAC31) and *Streptomyces thermocarboxydus* (0.9467±0.0058). Among 46 isolates, 14 (30.4%) isolates were positive for catalase production as they formed effervescence. From the obtained data and phylogenetic analysis we observed four genera, *Streptomycetes, Actinomycetes*, *Tsukamurella* and *Nocardiopsis*.Out of five plants selected for the present study, to our best knowledge endophytic actinomycetes were explored for the first time to our knowledge from *Mikania micrantha, Cassia fistula*and *Senecio scandens. Senecio scandens* has not been reported for any type of endophytic microbes and among these three plants maximum endophytic actinomycetes was recorded from *Mikania micrantha* (n=30, 34.48%).Best of our knowledge this is the first time reported that endophytic actinomycetes showing antifungal activity against *Fusarium udum.* Isolates BPSEAC40 and BPSEAC11 showed the most potent PGPR activities.Hence, these two isolates have potential to be used in agriculture sustainability developments.

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