# PLANT GROWTH PROMOTING ACTINOBACTERIA FROM RHIZOSPHERE SOILS OF BLACK PEPPER IN WAYANAD

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**(2018-11-091)**


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**By**

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**(2018-11-091)**

**THESIS**

***Submitted in partial fulfilment of the***

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**VELLANIKKARA, THRISSUR – 680 656**
**KERALA, INDIA**
**2021**

# DECLARATION

I, hereby declare that this thesis entitled **'Plant growth promoting actinobacteria from rhizosphere soils of black pepper in Wayanad'** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Date: 24-02-2021 (2018-11-091)

# CERTIFICATE

Certified that this thesis entitled **'Plant growth promoting actinobacteria from rhizosphere soils of black pepper in Wayanad'** is a record of research work done independently by **Ms. Rineesha Backer A.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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We, the undersigned members of the advisory committee of **Ms. Rineesha Backer A. (2018-11-091)**, a candidate for the degree of **Master of Science in Agriculture**, with major field in **Agricultural Microbiology**, agree that the thesis entitled **"Plant growth promoting actinobacteria from rhizosphere soils of black pepper in Wayanad"** may be submitted by **Ms. Rineesha Backer A.** in partial fulfilment of the requirement for the degree.

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***(Rineesha Backer A.)***

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| | |
|---|---|
| µm | Micrometer |
| µg $ml^{-1}$ | Micro gram per milli liter |
| BLASTn | Basic Local Alignment Search Tool |
| BNF | Biological Nitrogen Fixation |
| cfu $g^{-1}$ | Colony forming unit gram |
| cm | Centimeter |
| COA | College of Agriculture |
| $H_2SO_4$ | Sulphuric acid |
| NaOH | Sodium Hydroxide |
| DAI | Days After Inoculation |
| DNA | Deoxyribo Nucleic acid |
| h | Hour |
| min | Minutes |
| mg | Miligram |
| g | Gram |
| IAA | Indole Acetic Acid |
| N | Nitrogen |
| P | Phosphorus |
| K | Potassium |
| Zn | Zinc |
| kg | Kilogram |
| m | Meter |
| ml | Milliliter |
| mm | milli meter |
| mM | Millimolar |
| PGP | Plant Growth Promotion |
| pH | Hydrogen ion concentration |

| | |
|---|---|
| NCBI | National Centre for Biotechnology Information |
| No. | Number |
| rpm | Revolutions per minute |
| BV | Blank Value |
| TV | Titre Value |
| Trp | Tryptophan |
| ppm | parts per million |
| Sp | Species |
| rRNA | ribosomal Ribo Nucleic Acid |
| ZnO | Zinc Oxide |

# *1. Introduction*

# 1. INTRODUCTION

India is considered as 'The land of spices'. Black pepper (*Piper nigrum* L.), universally acclaimed as 'Black Gold' and also known as 'King of spices', is native to the tropical forests of Western Ghats of South India. This is one of the major export oriented spice crops of India, valued for its black and white berries, essential oil, oleoresin and other value added products. In India, black pepper is cultivated to a large extent in Kerala (94%) and Karnataka (5%) and to a limited extent in Tamil Nadu, Maharashtra, North Eastern states and Andaman and Nicobar islands.

India contributes 54 per cent area under pepper in the world but its production share is only 26.6 per cent. Other countries like Brazil, Indonesia and Malaysia contribute less area percentage, but larger share in total production. In Kerala, the crop is grown in about 82,761 ha with a production of 36,776 MT. In Kerala, Wayanad is an important pepper growing district with an area of 9939 ha and a production of 3123 MT (GoK, 2020).

Effective farming practices depend on extensive use of chemical fertilizers to enhance plant growth and yield. However, the prices, availability, environmental concerns and human health hazards due to the inclusion of these chemical fertilizers in food web are real issues of today's agriculture. Use of microbial inoculants as biofertilizers for reducing the use of chemical fertilizers, pesticides and other agrochemicals, without reducing the plant yield is currently a notable research area in agriculture, microbiology and biotechnology (Ahmad et al., 2008).

Plant growth promoting rhizobacteria (PGPR) are a group of naturally occurring, free-living soil bacteria that extensively colonize the rhizosphere or plant roots and improve plant growth, yield, soil fertility. PGPR, when applied to seed or crops, also confer tolerance to biotic and abiotic stresses (Vessey, 2003; Kumar et al., 2014). These microbes also have the ability to produce phytohormones such as indole acetic acid (IAA), gibberellins and cytokinins (Glick, 1995; Marques et al., 2010),

asymbiotic nitrogen fixation (Sahin et al., 2004; Khan, 2005) and solubilization of phosphorus minerals (Glick 1995, Joen et al., 2003). Indirect growth promotion occurs through the elimination of phytopathogens by the production of secondary metabolites such as hydrogen cyanide, siderophores, antibiotics, enzymes and fungicidal compounds (Dey et al.,2004; Lucy et al., 2004, Franco- correa et al., 2010).

Actinobacteria are Gram positive bacteria that are widely distributed in soil and also plant rhizosphere. They can degrade a wide range of biopolymers by secreting many hydrolytic enzymes and remain dormant in agricultural soils by forming spores (Alexander, 1977). To assess the diversity of actinobacteria in a particular ecosystem, isolation and characterization of actinobacterial strains are the basic requirements in order to exploit them for growth promotion and to enhance crop productivity (Deepa et al., 2010). However, in spite of high diversity of actinobacteria in soil, secondary metabolite production and their ability to survive under aggressive environments, actinobacteria have not been exploited for plant growth promotion, as compared to bacteria like *Pseudomonas* or *Bacillus* spp. (Doumbou et al., 2002).

Wayanad is a beautiful hill district situated in the Western Ghats, at 700 meters to 2100 metres above the mean sea level. The climate is sub-tropical to temperate and therefore this region is quite different from the rest of the State. No systematic studies have been carried out in this backward district, on native actinobacteria. In this context, the present study was taken up with the objective of isolation, screening and molecular characterization of actinobacteria from black pepper rhizosphere soils of Wayanad and evaluation of their plant growth promoting activities.

# 2. *Review of literature*

# 2. REVIEW OF LITERATURE

Black pepper (*Piper nigrum* L.) known as the 'King of the spices', is a flowering vine belonging to the family *Piperaceae*. The crop is cultivated for its fruit, known as a peppercorn, which is usually dried and used as a spice and for seasoning. Pepper is native to South Asia and Southeast Asia. Black pepper and its active ingredient piperine have potent antioxidant and anti-inflammatory properties (Butt et al., 2013). Clinical studies suggest that black pepper improves cholesterol levels, controls blood sugar, and maintains brain and gut health. One tablespoon (6 grams) of ground black pepper contains moderate amounts of vitamin K (13% of the daily value or DV), iron (10% DV) and manganese (18% DV), with trace amounts of other essential nutrients, protein, and dietary fibre (USDA, 2014).

According to FAOSTAT (2019), Vietnam is the world's largest producer and exporter of black peppercorns, producing 262,658 tonnes or 36% of the world total. Other major producers are Brazil, Indonesia, and India. Pepper production varies annually according to crop management, diseases, and weather. Vietnam dominates the export market, using almost none of its production domestically. In India, Kerala ranks first in black pepper production contributing about 97% of the total production. Wayanad is one of the main pepper growing tracts in Kerala with an area of 10,565 ha and a production of 4,136 tonnes (GoK, 2017). Black pepper production in Wayanad is declining year after year due to the poor soil health status, improper land management with changes in climatic factors leading to the incidence of biotic and abiotic stresses.

Another cause for low productivity of black pepper in India is the loss due to diseases and pests (Sarma et al., 1997). The important pathogen in pepper are *Phytophthora capsici* (the oomycete fungus) causing foot rot or quick wilt (Sarma et al., 1992; Kueh and Sim, 1992; Manohara et al., 1992). Another soil borne disease which is mostly seen in black pepper nurseries is basal wilt and the causal agent is *Sclerotium rolfsii*. This disease is characterized by rotting of basal region of the nursery plants (Anandaraj and Sarma, 1995). These pathogens are generally managed by the application of fungicides. Systemic fungicides like metalaxyl (1.25 g/L) and potassium

phosphonate (3 ml/L) are widely used for the control of *P. capsici* in black pepper (Ramachandran et al., 1991). Similarly, carbendazim (0.2%) or Bordeaux mixture (1%) is being widely used against *Sclerotium rolfsii*.

However, the non-judicious use of these chemical fertilizers and fungicides has resulted in environmental pollution, decreased diversity of non-target organisms and has also led to development of pathogen resistance (Bhandari, 2014). Effective farming practices, now a days, rely on extensive use of chemical fertilizers in order to enhance plant growth and yield. However, the cost, environmental concerns and the resulting human health hazards due to the inclusion of these chemical fertilizers in food chain are the major limiting factors. An increasing demand for the low-input agriculture has resulted in a greater interest in soil microorganisms which are able to enhance the plant nutrition, health and soil quality (Jeffries et al., 2003)

Because of these hazards, an alternative approach for plant growth promotion and crop protection is needed, which is less dependent on chemicals and is more eco-friendly. The demand for plant growth promoting (PGP) organisms is more because of the eco-friendly nature and large drive towards organic farming. There are several reports on the effective use of PGP organisms as a substitute for agrochemicals (Shimizu, 2000; Yang, 2008). The search for new potential agents yielded the actinomycetes, especially the genus *Streptomyces* as well the secondary metabolites derived from this actinobacterium, as promising tools for growth promotion and management of diseases (Behal, 2000; Hassan et al., 2011). Among the microbial groups, actinobacteria and arbuscular mycorrhizal (AM) fungi are known to promote activities which can improve agricultural developments (Barea et al., 2005). Actinomycetes are one of the major components of rhizosphere microbial populations and are useful in soil nutrient cycling as well as plant growth promotion (Merzaeva et al., 2012). During recent years, actinomycetes have attracted interest of scientists and researchers, as plant growth promoting agents, because of their ability to thrive under variable conditions (Shivlata and Satyanarayanan, 2015). The growth promotion is either by direct stimulation such as iron chelation, phosphate solubilisation, nitrogen fixation and phytohormone production or by indirect stimulation such as suppression of plant pathogens and induction of systemic resistance in host plants (Panhwar et al.,

2012). Research work carried out on plant growth promoting micro-organisms were mainly concentrated on bacteria and very limited on actinomycetes. Hence the present investigation was undertaken to identify potential actinomycetes with plant growth promotion ability from the rhizosphere of black pepper of Wayanad district and the literature pertaining to the study are presented in this chapter.

## 2.1 ACTINOMYCETES – AN INTRODUCTION

Actinomycetes are the most abundant organisms that form thread-like filaments in the soil and are responsible for characteristically "earthy" smell of freshly turned healthy soil (Bhatti et al., 2017). They are the major components of the microbial populations present in soil (Franco-Correa et al., 2016). These are found in plant rhizosphere (Suzuki, 2000). Das et al. (2008) described that the word "Actinomycetes" was derived from two Greek word "atkis" meaning "a ray" and "mykes" meaning a fungus" and they have characteristics of both bacteria and fungi. Actinomycetes belong to an extensive and diverse group of Gram-positive, aerobic, mycelial bacteria that play important ecological roles in soil nutrient cycling (Ames et al., 1984; Nonomura, 1989; Halder et al., 1991; Elliot and Lynch, 1995). According to De-Boer et al. (2005), they are well known for production of biologically active substances such as antibiotics, vitamins and enzymes and this increases their economic importance. They are also an important source of antimicrobial metabolites (Lazzarini et al., 2000; Basilio et al., 2003; Terkina et al., 2006). They are important not only to the pharmaceutical industries but also to the agriculture scenario, since they have the potential to inhibit the growth of several plant pathogens.

### 2.1.1 Habitat

Actinomycetes represent a ubiquitous class of microorganisms with wide distribution in various ecosystems of the world (Srinivasan et al., 1991). They exist in diverse habitats of the nature (George et al., 2012).

These organisms are primarily soil inhabitants (Kuster, 1968) but also found in a range of ecosystems like aquatic ecosystem, including the sediments collected from deep sea (Colquhoun, 1998 and Walker, 1975). Moncheva et al. (2002) and Diraviyam

et al. (2011) reported that actinomycetes were present even in extreme environments especially at cryophilic region Antarctica and even in desert soil.

### 2.1.2 Structure

Morphologically, actinomycetes resemble fungi because of their elongated cells that branch into filaments or hyphae. These hyphae can be distinguished from fungal hyphae on the basis of size, with actinomycete hyphae much smaller than fungal hyphae (Pepper and Gentry, 2015). According to Bhatti et al. (2017), the hyphae are generally without septum and under certain special conditions septa may be observed in some forms. The sporulating mycelium may be branching or non-branching, straight or spiral shaped. The spores can be spherical, cylindrical or oval. Initial microcolonies produced by them are composed of branching system filaments and after 24 to 48 h of production, they fragment into diptheroids, short chain and cocco-bacillary forms (Manulis et al., 1994). Goodfellow et al. (1998) reported that actinomycetes hava a rigid cell wall structure that helps to maintain the shape of the cell and prevents bursting of the cell due to high osmotic pressure.

Davenport et al. (2000) described that cell wall consists of a large variety of complex compounds including peptidoglycan, teichoic and teichuronic acids and polysaccharides. The peptidoglycan consists of glycan (polysaccharides) chains of alternating N-acetyl-d-glucosamine (NAG) and N-acetyl-d-muramic acid (NAM) and diaminopimelic acid (DAP), which is unique in prokaryotic cell walls. Teichoic and Teichouronic acids are chemically bonded to peptidoglycan. Shrijver and de Mot (1999) and Das et al. (2008), the chemical composition of actinomycete cell wall resembles Gram positive bacteria but because of their well-developed morphological (hyphae) and cultural characteristics, actinomycetes have been considered as a separate group from other common bacteria.

## 2.2 ACTINOMYCETES IN PLANT GROWTH PROMOTION

Actinomycetes of rhizospheric region of plants are known to produce active compounds, of which many are agro-important (Suzuki et al., 2000). This group of bacteria plays a variety of roles in the soil environment such as organic matter recycling, inhibition of soil borne pathogens in the rhizosphere and decomposition of complex

mixtures of polymers in dead plant, animal and fungal material which results in the production of various extracellular enzymes which are essential for crop production. Hamdali (2008) described the positive influence of actinomycetes on plants through multifunctional attributes like rock phosphate solubilization, siderophore production, antagonism against fungal phytopathogens *etc.* The important roles of the actinomycetes collected by the perusal of literature are discussed below.

### 2.2.1 Nitrogen fixation

Nitrogen is an essential element for promotion of plant growth because it has direct role in protein synthesis. Plant system is not capable of utilising the nitrogen present in the atmosphere. Hence, nitrogen fixation is needed for the conversion of atmospheric nitrogen to plant utilizable forms. The nitrogen fixing microbes converts the nitrogen gas present in the atmosphere to ammonia (Ahmed and Kilbert, 2014) with the help of enzyme Nitrogenase (Kim and Rees, 1994). Many of the soil actinomycetes of genera *Streptomyces, Frankia*, *Micromonospora* and *Nocardia* have symbiotic as well as asymbiotic associations with plant organs, especially roots. There are numerous evidences of actinomycetes involved in nitrogen fixation, such as *Frankia*. This is a widespread endophytic actinomycete symbiotically associated with plant roots and it fixes atmospheric nitrogen for host plants (Benson and Silvester 1993). Various studies reported that *Streptomyces* sp. have strong nitrogen fixing capability (Sellstedt and Richau, 2014). Majority of these species have a gene for nitrogen fixation (Dahal et al., 2017). *Streptomyces thermoautotrophicus* is capable of fixing nitrogen into biological form with the help of Molybdenum dinitrogenase and Manganese superoxide oxidoreductase (Sellstedt and Richau, 2013). In a study by Tokala et al. (2002), nitrogen fixation in roots of young pea seedlings with symbiotic *Rhizobium* sp. was observed to be promoted by *Streptomyces lydicus* strain WYEC108.

### 2.2.2 Phosphate solubilisation

Phosphorous is another important macronutrient for plant growth, next to nitrogen. Phosphorous remains unavailable to plants due to its complex nature and phytate state. Organic phosphate present in the soil is mineralized by the phosphatase enzyme, which is excreted by some microorganisms and is released for the utilisation of plants (Gerretson, 1948). Actinomycetes are of special interest in this aspect since

these filamentous bacteria are capable of colonization in the root tissue and producing spores for their survival in agricultural soils. These microbes play a key role by releasing a soluble phosphate from insoluble rock phosphate (Hamdali et al., 2008). Actinomycetes have also been reported as potential candidates for solubilising the insoluble organic and inorganic phosphorus compounds. Very few studies have been carried out and Sahu et al. (2006) demonstrated phosphate solubilising potential of actinomycetes in the estuarine environment.

Actinobacteria belonging to *Saccharopolyspora, Thermobifida, Thermomonospora, Micromonospora, Nocardia, Actinomadura, Rhodococcus, Actinoplanes, Microbispora* and *Streptosporangium* produce phosphatase enzymes which have been classified according to their alkaline or acid activity, depending on reaction conditions. Phosphate solubilisation does not involve acidification of the medium for the growth in the case of *Streptomyces* sp. (Rungin et al., 2012). Nimnoi et al. (2010) also reported the production of siderophore and phosphate solubilisation of actinomycetes. Various genera of actinomycetes such as *Rhodococcus, Arthrobacter, Streptomyces, Gordonia* and *Micromonospora* were reported to have phosphate solubilization potential under laboratory and glasshouse conditions (Jog et al., 2014). Under P-deficient soils, *Streptomyces griseus*, *Streptomyces* spp., *Micromonospora aurantiaceae* performed well in terms of P-solubilisation under wheat crop (Hamdali et al., 2008; Jog et al., 2014). Production of various organic acids such as gluconic, citric, malic, lactic, propionic, oxalic and succinic acids by actinomycetes is believed to be the mechanism of their phosphate solubilization (Hamdali et al., 2010; Jog et al., 2014). The root exudates represent the major source of nutrients, such as carbohydrates, organic acids, amino acids and they influence the diversity of phosphate solubilizing microbes and their capacity with respect to different rhizosphere of plant. *Streptomyces thermolilacinus, S. youssoufiencis, S. rochei, S. carpinensis* were the best phosphate solubilisers reported among *Streptomyces* spp.

### 2.2.3 Potassium solubilisation

Potassium is an inevitable element for growth and development of plants, which is commonly supplemented through fertilizers. Apart from growth promotion aspects, it also has significant role in processes like photosynthesis, enzyme activation and

protein synthesis (Armengaud et al., 2009; Basak and Biswas, 2008; Lin, 2010). Liu et al. (2010) reported the ability of *Streptomyces* sp. to release potassium from silicate minerals and they also found the capacity to degrade potassium bearing rocks by solid state fermentation.

### 2.2.4 Zinc solubilisation

The microelement zinc plays a vital role in various metabolic processes in plants, and its deficiency adversely affects the growth and development of crop plants (Sharma et al., 2012). The results obtained by Patel and Thakker (2020) revealed that zinc mineralization by a strain YM4 recorded a solubilisation index of 76.31. ZnO (0.1%) supplemented in agar plate was solubilized by strain YM4, which was clearly observed surrounding the colony diameter in the form of zone of clearance. Zinc mineralization was estimated quantitatively in broth supplemented with 0.1% ZnO as Zn source. After incubation of 10 days, culture supernatant was analysed under atomic absorption spectroscopy (AAS), which indicated that strain has the capacity to solubilize Zinc.

### 2.2.5 Production of plant growth regulators

Plant Growth Regulators (PGR) are known as plant hormones. PGR are small molecules that affect plant growth and development at very low concentrations (Garcia-Breijo, 2003). Actinomycetes are known to produce these growth regulators. IAA producing *Streptomyces* spp. improves plant growth by increased seed germination, root elongation and root dry weight on peas (Tokala et al., 2002), beans (Nassar et al., 2003), tomato (El-Tarabily, 2008), wheat (Sadeghi et al., 2012), chilli (Passari et al., 2015) and rice (Gopalakrishnan et al., 2013). Several species of *Streptomyces* such as *S. nobilis, S. kunmingensis, S. olivaceoviridis, S. rimosus S. rochei, S. griseoviridis, S. lydicus, S. djakartensis, S. griseorubens, S. globisporus, S. caviscabies and S. enissocaesilis* (Mahadevan and Crawford, 1997; Gopalakrishnan et al., 2011; Anwar et al. 2016) have been reported to produce PGR. (Khamna et al. (2009) reported members of the genus *Streptomyces* having indole acetic acid (IAA) producing potential which restricted fungal phytopathogens like *Alternaria brassicicola* (rose apple anthracnose), *Colletotrichum gloeosporioides* (potato dry rot), *Fusarium oxysporum* (chinese

cabbage leaf spot), *Penicillium digitatum* (orange green mold) and *Sclerotium rolfsii* (damping-off of balsam) exhibiting reduced disease symptoms.

Similarly, Merckx et al., (1987) reported the involvement of actinomycetes in plant growth promotion through IAA production as well as siderophore production, resulting in enhanced nutrient uptake. Rhizospheric actinobacteria possess the ability to produce IAA, cytokinins and GA3 (El-Tarabily and Sivasithamparam, 2006; Vijayabharathi et al., 2016). Nimnoi et al., (2010) reported the production of IAA and ammonia, a trait of plant growth promotion by endophytic actinomycetes from eaglewood (*Aquilaria crassna*). Carvalho et al. (2017) also reported the production of IAA by actinobacteria.

*Streptomyces flavus* producing cytokinins were isolated from rhizospheric soils by Coppola and Giannattasio (1968). Another *Streptomyces* sp. isolated from actinorhizal plant, *Ochetophila trinervis* showed significant production of IAA, gibberellic acid and zetaine (Solanset al., 2011).

**2.2.6 Antagonistic activity**

Actinomycetes are commonly used as biocontrol agents against different plant pathogenic microorganisms like *Alternaria* sp. (Chattopadhyay and Nandi, 1982), *Rhizoctonia* sp. (Merriman et al., 1974; Rothrock and Gottlieb, 1984), *Verticillium* sp. (Wadi and Easton, 1985), *Fusarium* sp. (Sabaou and Bounaga 1987), and *Macrophomina* sp. (Hussain et al., 1990). Members of the genus *Streptomyces* have proven their extensive biocontrol potential of plant diseases (Emmert and Handelsman, 1999; Weller et al., 2002). Patil et al. (2010) demonstrated the functioning of *Streptomyces* spp. as an antagonist against *R. solani* with respect to the disease suppression potential. They revealed that the mechanism behind the suppression of the pathogen was induced systemic resistance through increased accumulation of phenolic compounds as well as phenyl alanine ammonia lyase in tomato plants, when grown under greenhouse conditions. The members of the genus *Streptomyces* were also exploited commercially for plant disease control. In China, *Streptomyces sp.* strain 5406 had been used in cotton for more than 30 years for crop protection against soil-borne pathogens as reported by Yin et al. (1965) and Valois et al. (1996). Similarly, the company Kemira Agro-Oy had formulated biofungicides with live *Streptomyces*

*griseoviridis* as component to control *Fusarium* and *Alternaria* infections (Lahdenpera et al. 1991).

Perusal of literature revealed that different mechanisms are involved in plant disease control by actinomycetes. The various mechanisms are inhibition of the pathogen by antimicrobial compounds (antibiosis), competition for iron through production of siderophores, competition for colonization sites and nutrients supplied by seeds and roots, induction of plant defence mechanisms, inactivation of pathogen germination factors in seed or root exudates, degradation of pathogenicity factors of the pathogen such as toxins, parasitism that may involve production of extracellular cell wall degrading enzymes like chitinase and β-1, 3 glucanase capable of lysing pathogen cell walls (Keel and Defago, 1997; Whipps, 1997). All these mechanisms or interactions of antagonism act either individually or synergistically by actinomycetes and none of the mechanisms were mutually exclusive and several modes of actions could be exhibited by a single actinomycetes strain for the suppression of plant disease (Patil et al., 2010).

**2.2.4.1 Competition**

In soil, competition for resources such as nutrients, oxygen and colonization site occurs generally between diverse soil-inhabiting organisms. It seems to be a biocontrol mechanism when an antagonist directly competes with pathogens for the above mentioned resources, where actinomycetes dominate due to their strong physiology, biodegradation, biosynthetic and antagonistic potential. There are several modes of competition such as root inhabiting microorganisms, which are having prime competition for infection sites at the root surfaces, while competition for nutrients, especially for carbon, results the inhibition of fungal spore germination in soil (Alabouvette et al., 2006). Similarly, actinomycetes can compete for trace elements, such as iron, copper, zinc, manganese etc., in soils due to their heavy metal degradation potential.

Siderophores produced by actinomycetes are low molecular weight compounds with high iron affinity which competitively acquire ferric ion by chelation. According to Haas and Defago (2005), siderophores can function as diffusible bacteriostatic or fungistatic antibiotic by creating iron deficiency. Although several authors have demonstrated the contribution of siderophores to disease suppression in certain

circumstances; it is believed that siderophores alone are not sufficient to account for suppression; if they were, it would be difficult to explain why most strains which produce siderophores, do not have biocontrol activity (Haas and Defago 2005) and hence there is need of synergists to accomplish the task.

**2.2.4.2 Parasitism**

This phenomenon involves physical interaction between both antagonists and pathogen, which results in death of pathogen due to destruction of the cell wall, where probabilities tend to point out at hydrolytic enzymes produced by bioagent (Adams, 1990). Chitin, cellulose and ß-1,3-glucan are the major structural components of most fungi, which get denatured by extracellular enzymes such as chitinase, cellulase and ß-1,3- glucanases. It was reported by Dunne et al. (2000) that overproduction of extracellular protease in the mutant strain of the bacterium *Stenotrophomonas maltophilia* W81 resulted in improved biocontrol of *Pythium ultimum*. Sun et al. (2006) reported reduced tomato root gall disease severity index by 13.4–58.9% compared to control, through parasitic activity of four actinomycetes strains. In the same context *Streptomyces* has also been shown to play an important role in mycoparasitism of phytopathogenic fungi based on their chitinase and glucanase producing potential (Gomes et al., 2000; Whipps 2001).

**2.2.4.3 Antibiosis**

Antibiosis refers to the inhibition or destruction of the pathogen by metabolites produced by the antagonist and includes volatile compounds, toxic compounds, antibiotics, etc. which are deleterious to the growth or metabolic activities of other microorganisms at low concentrations (Fravel, 1988). Among all microorganisms, actinomycetes are leading with big contribution in producing different classes of antibiotics (Andrade et al., 1994). Getha and Vikineswary (2002) demonstrated that biological control of *Fusarium oxysporum* f. sp. *cubense* by *Streptomyces violaceusniger* strain G10 was through antibiosis. There are plentiful compounds *viz*. 2,4-diacetylphloroglucinol (2,4-DAPG), pyrrolnitrin (PRN), pyoluteorin (PLT) and different derivatives of phenazine (Phz), that participated actively and have been implicated successfully in biological control of phytopathogens.

### 2.2.4.4 Lytic Enzyme Production

Actinomycetes produce several enzymes such as protease, cellulase, amylase, gelatinase, lectinase, catalase, chitinase and urease, which degrade complex organic materials (Gulve and Deshmukh, 2012). The enzymes are known to exhibit hyperparasitic activity by attacking pathogens by excreting cell wall hydrolases. Chernin and Chet (2002) revealed that chitinase produced by *Streptomyces* sp. strain 385 was principally involved in antagonism through various modes of action such as inhibition of spore germination and germ tube elongation and restriction of growth by digesting and lysis of fungal mycelia. However, in some of the actinomycete strains, chitinolytic activity appears less essential as in case of *Streptomyces.* This actinobacterium preferably synthesises $\beta$-1, 3-glucanase and lyses fungal cell wall of *F. oxysporum* f. sp. *cucumerinum* (Singh et al., 2009). There are β -1, 3- glucanases and β -1, 4- glucanases (Bielecki and Galas 1991; Gilbert et al. 1995). However, Valois et al. (1996) reported β-1, 6- glucanase activities associated with actinomycetes. The regulation of lytic enzyme production involves GacA/GacS regulatory systems, which overlap with the regulation of other microbial metabolites like siderophores and antibiotics in *Pseudomonas* spp. (Corbell and Loper 1995; Gaffney et al. 1994; Natsch et al. 1994; Sacherer 1994). Bouizgarne and Aouamar (2014) also reported the production of proteases by actinobacteria.

### 2.2.4.5 Deactivation of Virulence Factors

Another biocontrol mechanism is deactivation of pathogen virulence factor, such as detoxification of albicidin toxin produced by *Xanthomonas albilineans* (Zhang and Birch 1997), which includes reversible as well as irreversible detoxification. In the former mechanism, binding protein is synthesized and it binds reversibly to inactivate the toxin, while irreversible detoxification is mediated by an esterase (Zhang and Birch 1997). In another case, fusaric acid, a phytotoxin produced by *Fusarium* was also hydrolyzed by several microorganisms (Thomashow and Weller 1996). In a simultaneous approach, certain PGP bacteria quench pathogen quorum sensing capacity by degrading auto inducer signals, thereby blocking the expression of numerous virulence genes (Uroz et al. 2003), and hence, this approach holds tremendous potential for alleviating disease, even after the onset of infection, in curative manner.

### 2.2.4.6 Induction of Plant Defence System

Plant growth promoting bacteria are known to induce non-specific plant defence mechanisms known as induced systemic resistance and systemic acquired resistance. *Streptomyces* spp. induced plant defence and resistance mechanisms against *F. oxysporum* and *E. carotovora* (Conn et al., 2008). They also reported systemic acquired resistance pathways and jasmonate or ethylene pathways. Lehr et al. (2008) reported induction of systemic resistance by *Streptomyces* spp. against pathogenic fungus *Heterobasidion abietinum.* Zhao et al. (2012) reported a novel isolate of *Streptomyces gamaensis* having antifungal activity against fungal pathogens such as *Botrytis cinerea*, *Heterobasidion abietinum* and *Fusarium oxysporum* f. sp. *cucumerinum*.

## 2.3 CHARACTRERISATION OF ACTINOMYCETES

For the identification of potential actinomycetes with plant growth promoting and biocontrol abilities, various characterisation techniques are available. Perusal of literature revealed various studies with respect to the isolation and characterisation of actinomycetes.

### 2.3.1 Isolation of actinomycetes

Soil samples for the isolation of actinomycetes have to be taken from a depth of one cm surrounding the root system of plant after removing 3 cm top soil and have to be collected in polythene bags. The samples have to be brought to the laboratory and stored at 4° C for further study (Thampi and Bhai, 2017). Matsukawa et al. (2007) and Hong et al. (2009) suggested air drying of the samples for one week and sieving through 2 mm pore sieve. The pre-treatment of the soil enhances the isolation of actinomycetes by destroying most of the unwanted microbial population. Ten grams of each sample were suspended in 90mL of sterile physiological saline (0.85% NaCl in distilled water) in a bottle and kept for shaking on an orbital shaker (at 100 rpm) at 28 ± 2 ◦C for 1h. At the end of shaking, the samples were serially diluted up to $10^5$ dilutions and samples from $10^4$ and $10^5$ dilutions were spread plated (0.1mL) on actinomycetes isolation (AIA) agar (HiMedia Laboratories, Mumbai, India) and incubated at 28 ± 2 ◦C for 7 days (Sreevidya et al., 2017).

For the isolation, Suarez-moreno et al., (2019) removed plants roots from the soil and subsequently hand-shaked for 10 min to remove the bulk soil. The remaining

adhering soil was considered as rhizospheric soil, and was collected by hand-shaking roots for 10 min in a sterile plastic bag. Rhizospheric soil samples (4–5 g for each) were then mixed with 1 g of $CaCO_3$. Samples were further dried at 45°C for 1 h (Gurung et al., 2009). Actinobacteria were subsequently isolated by spread plate technique following the serial dilution of soil samples on starch casein agar (Starch 10 g/L, Casein 1 g/L, $K_2HPO_4$ 0.5 g/L, Agar 13 g/L) and incubated at 30°C for a week.

**2.3.2 Cultural characterisation**

Cultural characteristics of actinomycetes include colour, texture, growth rate, pattern of growth, sporulation, pigmentation and colour on the back side of Petri plates. Sreevidya et al. (2016) investigated the cultural characteristics of some selected isolates of actinomycetes by streaking them on Actinomycetes Isolation (AI) agar by quadrant streaking technique and incubated for 5 days at 28 ± 2 ◦C. At the end of incubation, the isolated colonies were observed for their morphology. Thampi and Bhai (2017) studied this by growing the actinomycetes in different ISP medium (Shirling and Gottlieb, 1966) at $28^0$C for 7 days. They observed the characters such as colony appearance, type of areal hyphae and growth of vegetative hyphae and the colour of spore masses and diffusible pigment production were visually estimated with the help of RHS-colour code (RHS colour chart, Fifth edition-Royal Horticultural Society).

**2.3.3 Morphological characterisation**

The various microscopic observations like colour of hyphae, branching pattern, septation, presence of conidia, conidial septation, types, shape and size of spores, length and breadth of spores and presence of sexual structures are useful. Spore chain morphology of actinomycetes was studied by Hopwood (1960) using modified cover slip culture method. The isolates were inoculated on PDA agar block, a sterile cover slip was placed over it and incubated at 28 C for 3 days. The mycelium grown on the cover slip was later stained with crystal violet and the spore chain morphology was observed by light microscopy (100X magnification). Petrolini et al. (1986) studied the spore surface ornamentation and morphology using SEM (Hitachi SU 6600 FESEm). For the observation they took the inoculum plugs (8 mm diameter) from cultures grown on ISP-2 agar, incubated at 30 C for 5 days and were then fixed with 2.5% glutaraldehyde for 2 h. These plugs were then dehydrated in a graded alcohol series

(30–95%) for 10 min each followed by acetone series (30–95%) for10 min, and finally critical point drying.

**2.3.4 Biochemical characterisation**

Various biochemical tests performed for actinomycetes are starch hydrolysis, triple sugar iron test, fermentation of citrate for the identification of the potent isolates (Abbas, 2006).

Varghese et al. (2012) studied biochemical characteristics of 36 actinomycete strains isolated from the shola soils, a relatively unexplored biodiversity hotspot of tropical montane forest. Ability of actinomycete isolates to ferment and produce acids from different carbohydrate sources like innositol, mannose, sorbitol, galactose, mannitol, xylose, rhamnose, arabinose, lactose and fructose were studied by them. The results revealed that almost all the carbon compounds were utilized by one or another actinomycete isolate and the most preferred carbon sources were found to be xylose (94.44%) followed by fructose and mannose (91.66%) for the actinomycetes. Only 41.76 per cent of the isolates were able to ferment lactose. They also found out that ability of actinomycetes differ considerably among the isolates for the decomposition of protein and amino acid. About 72.22 per cent of the isolates were capable of decomposing milk protein casein and 61.11 per cent of the isolates decomposed tyrosine. Only 8.33 per cent of the strains were able to decompose amino acid hypoxanthine and none of them were able to decompose amino acid xanthine. Potential of the actinomycete isolates to reduce esculin, urea and hippurate and to resist lysozyme was also checked by them. About 91.66% of the isolates showed ability to decompose esculin and 63.88% of the isolates had the capacity to produce urease and to decompose urea. Only 25% of the isolate were able to decompose hippurate and 94.44% showed lysozyme resistance.

Sheik et al. (2017) characterized the actinomycete isolates by various biochemical tests such as indole test, methyl red test, vogus-proskauer test, citrate utilization test, triple sugar iron test, nitrate reduction test, starch hydrolysis test, catalase test, mannitol and sucrose utilization tests. They observed that indole production was strictly negative, methyl red was positive for all except DOM1, Voges-Proskauer test was negative for all the isolates, citrate was positive was for all except DP3. TSI was negative for all the isolates, nitrate reduction was positive for all the

isolates except DOM3, starch hydrolysis was positive for all the isolates and catalase was also positive for all the isolates. Mannitol utilization was positive for DP3 and DP4. Sucrose utilization was positive for all the isolates except DP3. After observation of cultural, morphological, physiological and biochemical characteristics it was confirmed that these isolates belong to the species of the genus, *Streptomyces*.

**2.3.5 Molecular characterisation**

Thampi and Bhai (2017) used 16S rRNA gene sequencing to identify three promising isolates *i.e.* IISRBPAct1, IISRBPAct25, and IISRBPAct42 at the species level. Total genomic DNA of the isolates was extracted by following modified protocol of Kutchma et al. (1998). Comparative analysis of 605 nucleotide position of 16S-rRNA gene sequence of the isolate IISRBPAct1 showed that the strain was closely related to the members of *Streptomyces* species viz., *S. chattanoogensis*, *S. albulus*, *S. lunalinharesii, S. diastatochromogenes, S. ahygroscopicus*, and *S. albogriseus* with 99% similarity. IISRBPAct25 was closely related to *S. rimosus*, *S. chrestomyceticus*, *S. wuyuanensis*, *S. platensis*, *S. bingchenggensis*, and *S. auratus* with 98% similarity while IISRBPAct42 showed 97% similarity to *S. olivaceiscleroticus, S. albofaciens, S. niger, S. ochraceiscleroticus, S. erumpens, S. purpurogeneiscleroticus* and *S. griseocarneus* with 98% query coverage.

Total genomic DNA from actinobacteria was extracted by Franco-Correa et al. (2010) using a modified protocol from Sanjuan (Com. Per) and 16 S rRNA sequencing was carried out to identify the isolates at species level. They confirmed that as in the case of other bacteria, the 16S rRNA gene sequencing approach is routinely used to identify actinomycete isolated after comparing with already described organisms in an rDNA sequence database (Edwards, 1993; El-Tarabily and Sivasithamparam, 2006). The F1 and R5 primers, used for amplification of 16S rDNA gene of the actinomycete isolates confirmed their efficiency to amplify all sequences of 16S rDNA gene of target bacteria (Cook and Meyers, 2003).

**2.3.6 Functional characterisation**

**2.3.6.1 Nitrogen fixation**

Biological nitrogen fixation of actinobacteria was confirmed by Acetylene Reduction Assay (ARA) (Hardy et al., 1968). For this purpose, 100 µL of washed cells were inoculated into 17 mL of semisolid NFb medium in 25 mL serum bottles with a

cotton cap, and cultures were maintained at 30°C, without agitation (Dobereiner, 1995). After 5 days, 2 mL of acetylene were injected into the flask and a rubber stopper was used to replace the cotton cap. The acetylene and ethylene concentrations of each sample were determined by gas chromatography, using a chromatograph Varian 3400-G-crom, equipped with a flame ionization detector and a capillary column (Hayesep Porapak N 80/100 column; 6′ X 1/8″). Ethylene and acetylene estimation was done by integrating the area under the curve for each compound, at retention times of 1.417 and 2.40 min, respectively.

**2.3.6.2 Phosphate solubilizing ability**

Phosphate solubilization capacity of the actinomycete isolates was determined by inoculating on Pikovskaya agar containing tricalcium phosphate and incubating at 28$^{o}$ C for 5–7 days. Mehta and Nautiyal (2001) described that formation of a clear zone around the colonies, which indicated phosphate solubilization by the isolates. Similarly, phosphate solubilizing abilities were qualitatively determined by Saurez-moreno et al., (2019) by inoculating single colonies of each strain in National Botanical Research Institute's Phosphate growth medium (NBRIP) (Nautiyal, 1999) and SRS media (Sundara-Rao and Sinha, 1963). Plates were incubated at 30°C for 5 days, and colonies with a clear orange halo in NBRIP media and/or a purple colour in SRS media were marked positive for phosphate solubilization. A quantitative determination was performed by growing each strain in 100 ml of NBRIP liquid media supplemented with 5 g/L $CaCO_3$, for 5 days. Subsequently, the solubilized phosphate present in the supernatant was measured with the Spectroquant Phosphor Kit (Merck).

**2.3.6.3 Zinc solubilisation ability**

Zn solubilization of actiomycetes was determined in mineral salts agar medium amended with 0.1% of insoluble zinc oxide. The 5 mm bit of actively growing culture were inoculated to medium and incubated for appearance of clear zone around the colonies up to 15 days (Venkatakrishnan et al., 2004). In an investigation conducted by Alagappan and Govindaraju (2016), there were 143 Zn solubilizing bacterial isolates obtained from rice rhizosphere soil samples using Tris-mineral salt growth medium supplemented with insoluble source of zinc such as Zinc Oxide (ZnO) and Zinc Carbonate (ZnCO3) individually. Among the ZSB isolates, maximum zinc solubilizing halo zone was observed with the isolate AGM3 followed by AGM9 both on ZnO and

ZnCO3 amended solid tris-mineral salt growth medium with the diameter of 13.21 mm, 10.71 mm and 11.74 mm, 7.90 mm respectively. Similarly, in broth assay conducted by them, the AGM3 showed high value of zinc solubilization than AGM9 in both ZnO and ZnCO3 supplemented medium with a value of 36.54 µg Zn $ml^{-1}$, 35.40 µg Zn $ml^{-1}$ and 33.14 µg Zn $ml^{-1}$, 32.69 µg Zn $ml^{-1}$ respectively. The SEM images of AGM3 exhibited better solubility of ZnO than ZnCO3. Both AGM3 and AGM9 bacterial isolates were confirmed as *Acinetobacter* sp. through biochemical tests and 16S rRNA gene sequence analysis. In pot experiment, among the all treatments, the combined use of AGM3 and AGM9 with ZnO and ZnCO3 resulted in maximum plant growth. The assay of fractionation of soil zinc after harvest showed an increase in exchangeable zinc, manganese oxide, bound zinc, crystalline and amorphours bound zinc and a decrease in organically complexed and carbonate bound zinc compared to untreated control.

**2.3.6.4 Siderophore production**

Siderophore production by actinomycetes was studied by inoculating the cultures grown on yeast malt extract (YM) agar to CAS-substrates with modified Gaus No. 1 (MGs-1) medium. The colonies which utilize iron present in the media produce orange zones around them and are considered as siderophore-producing isolates (Schwyn and Neilands, 1987). Thampi and Bhai (2017) reported that siderophore production was observed in all the three potential isolates of *Streptomyces* (IISRBPAct 1, IISRBPAct 25 and IISRBPAct 42) isolated from the black pepper tracts of Kerala and Karnataka. Siderophore production can also be determined in Chrome Azurol Blue agar (Schwyn and Neilands, 1987) or be modified to use KOH to adjust the pH as suggested by Mahmoud and Abdallah, 2001.

**2.3.6.5 ACC deaminase production**

1-aminocyclopropane-1-carboxylate (ACC) deaminase production was determined by Belimov et al. (2001) and El-tarabily (2008) for investigating the ability of actinomycete strains to use ACC as sole nitrogen source. For this purpose, single colonies were inoculated in Dorwin-Foster media (DF) supplemented with 3 mM ACC as sole nitrogen source, or with 2 g/L $(NH_4)_2SO_4$ as growth control.

#### 2.3.6.6 Extracellular enzyme production

Chitinase production by actinomycetes was estimated by Hsu and Lockwood by amending agar plates with colloidal chitin suspension and mineral salts. Cellulolytic activity was evaluated by spotting 2 µL of 5-day old cultures on the surface of CMC-media plates, in which CMC was the sole carbon source. Cultures were incubated at 30°C for 4 days, and then strains were assayed for their ability to degrade CMC by flooding each plate with a 0.2% Congo red solution for 15 min, followed by washing with 1 M NaCl. Activity halo dimensions were then measured from the border of the colony to the outer edge of the halo (Farkas et al., 1985). Similarly, ligninolytic activities were determined in guaiacol containing media and positive activity was scored when a red halo was formed around colonies, due to the oxidation of the substrate to tetraguaiacol (Nagadesi and Arya, 2012). The potential isolates were screened qualitatively for the production of enzymes such as amylase, protease, lipase and cellulase. Standard methods were given for the assay of these enzymes; amylase (Mishra and Behera, 2008), protease (Manachini et al., 1988) and lipase (Gulati et al., 1997).

#### 2.3.6.7 Bio control efficiency

Bhai et al. (2011) and Sutthinan et al. (2009) evaluated the actinomycetes for their activity against major pathogens of black pepper *viz.*, *P. capsici*, and *S. rolfsii* by dual culture technique Single colonies of actinomycetes were streaked on both sides of the Potato Dextrose Agar (PDA) plate at a distance of 2 cm away from the periphery of the plate. After 5 days, 5 mm mycelial plugs taken from the edge of 72 h old culture of the target pathogen was inoculated at the centre of the plate in between the actinomycete streaking and incubated at 26 C for 4–5 days. The *in vitro* inhibitions by actinomycetes were recorded by measuring the radial growth of the pathogen and percentage inhibition was calculated (Thampi and Bhai, 2017).

### 2.4 *In vivo* STUDIES

Numerous studies (pot culture as well as field evaluation) were conducted to isolate actinomycetes from the rhizosphere and to evaluate their efficiency in plant

growth promotion. These experiments attempted to identify potential actinomycete candidates for effective utilisation in plant growth promotion and disease management.

### 2.4.1 Pot culture experiments

*Streptomyces* sp. strain DBT204 isolated from tomato showed plant growth promotion in seedlings of both chilli and tomato (Passari et al., 2016). Genes *iaaM* and *acdS* were amplified by them and phytohormones like indole acetic acid, kinetin, and six antibiotics were detected and quantified. They have also reported endophytic *Streptomyces* sp. strain BPSAC 34 and *Leifsonia xyli* strain BPSAC 24 isolated from ethano-medicinal plants of Mizoram, which led to maximum increase in shoot and root length of chilli plant when used as a mixture (Passari et al., 2015).

Rhizosphere competent 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase- producing *Streptomycetes filipinensis* and *S. atrovirens* strains isolated from tomato rhizospheric soil exhibited plant growth promoting traits. *Streptomycetes filipinensis* no.15 was superior in PGP performance on tomato compared with S. atrovirens no.26, as it also produced IAA along with ACC deaminase activity which enhanced plant growth by reducing endogenous ethylene through *in planta* ACC (El-Tarabily, 2008).

The study conducted by Chukuwuneme (2019) has shown that the inoculation of drought tolerant *S. pseudovenezuelae* (MG547870) and *A. arilaitensis* (MG547869) increase plant growth and reduce the undesirable effect of drought stress, when used as bioinoculants on maize plant. *Streptomyces pseudovenezuelae* and *Arthrobacter arilaitensis* with high IAA and ACC production mitigated the impact of drought on the maize plants and increased plant biomass and physiological parameters.

Syiemiong (2019) isolated actinomycetes from a limestone mining soil located at Mawsmai in Meghalaya. Thirty-five isolates were found to have at least one potential PGP property and three isolates had two out of the four PGP properties tested. *Streptomyces* was the dominant PGPA with twenty-nine isolates out of the thirty-five isolates projected. The other PGP isolates were three *Nocardia* isolates, one *Actinomadura* isolate, one isolate belonging to the *Rhodochrous* group and one unidentified non-Streptomycete isolate. The projected PGPA isolates reported in this

work were comparable in their PGP ability to the PGPA reported by other workers. Some of the best performing PGPA isolates from this work warrant further investigations *in vivo* in a plant-actinobacterium interaction set-up.

Sreevidya et al. (2016) isolated and characterized actinomycetes with plant growth-promotion, from the rhizosphere of chickpea. A total of 89 actinomycetes were screened for their antagonism against fungal pathogens of chickpea by dual culture and metabolite production assays. Four most promising actinomycetes (SAI 13, SAI29, VAI 40, VAI 7) were evaluated for their physiological and plant growth-promotion properties under *in vitro* and *in vivo* conditions.

Thirty actinomycete strains were isolated from the rhizosphere of field-grown plants (*Trifolium repens* L.) by Franco-Correa et al. (2010). These strains were characterized and identified by microscopic characteristics, biochemical tests and molecular techniques. The 16S rDNA sequence analysis showed that most of the isolates belong to the *Streptomyces* genus. These thirty isolates were tested for their capabilities of solubilizing/mineralizing sparingly phosphate sources, $N_2$-fixation and/or siderophore production, typical traits of PGPR. Phosphate solubilizing ability was widely exhibited by the isolates. All of them produced acid phosphatase and thirteen of them alkaline phosphatase. Ten strains grew in N-free media. Almost all strains produced siderophores, however the production level was very low and only the strain *Thermobifida* (MCR24) released considerable amounts of this metabolite. *Streptomyces* (MCR9), *Thermobifida* (MCR24) and *Streptomyces* (MCR26) were selected to test their interactions with arbuscular mycorrhizal (AM) fungi because they produced the highest plant growth beneficial effects among ten isolates preselected as promissory PGPR.

Jiang and Song (2013) separated and purified 87 strains of actinomycetes from soil by dilution plate separation method. With pepper anthracnose (*Colletotrichum gloeosporioides*) as indicator pathogens, these actinomycetes were subjected to primary and secondary screening by cross streak method and mycelial growth inhibition method respectively. Finally, three actinomycetes with obvious antimicrobial activity were separated by them.

Hoai et al. (2018) isolated microorganisms from rhizospheres of pepper in Tay Nguyen and screened beneficial microbes against two pathogenic fungi *Fusarium oxysporum* and *Rhizoctonia solani* using agar well diffusion assay. The results showed that there was a difference in microbial density among the samples collected from diseased and healthy pepper. They selected and purified 391 strains including 236 bacteria, 149 actinomycetes and 6 fungi for screening antifungal activity. Out of isolated microorganisms, 44 strains (36 bacteria, 6 actinomycetes, and 2 fungi) showed antagonistic activity against at least one of two pathogens (*F. oxysporum* and *R. solani*). Identification of isolates with highest activity using the 16S rRNA gene sequences showed that the actinomycetes belonged to *Streptomyces* sp. and *Streptomyces diastatochromogenes*.

Pattanapipitpaisal and Kamlandharn (2012) isolated 283 strains of actinomycetes from rhizoshere-associated soils from Thailand using Enrichment Media (EMCA agar) for isolation of chitinase-producing actinomycetes. All strains were screened for chitinolytic activity and sixty-eight strains gave significant clear zone on EMCA agar plates. The selected chitinolytic strains were assayed for *in vitro* antagonism against *Sclerotium rolfsii* using corn meal agar (CMA agar) assay procedure and the result showed that thirteen isolates had remarkable ability for inhibiting the growth of the fungus. Two strains (PACCH24 and PACCH225) of antagonists reduced the disease development at 90 per cent.

Xuan et al. (2018) isolated and identified potential bio-control agents of actinomycetes to manage phytophthora blight in pepper. About 108 strains of actinomycetes were tested for antagonism against *Phytophthora capsici* by observing the disease control and plant growth promotion value under greenhouse condition. The effect of the population of microbes in the rhizosphere of pepper was studied prior and after inoculation of antagonistic actinomycetes. Results of the screening revealed that two strains (0108 and 0110) were found superior *via* sterile soil and natural soil in pot experiments and showed better control effect to phytophthora blight of pepper. These two strains showed relative control efficiency of 75 per cent and 81 per cent respectively in the $14^{th}$ day. The efficiency were reduced to 65 per cent and 56 per cent respectively in the 21 days after inoculation of the two strains 0108 and 0110. These two strains

heavily impacted the soil microorganism population to control the development and reproduction of *Phytophthora capsici* in different part of pepper roots and reduced the infection of *Phytophthora capsici* in the pepper roots, and promote the growth of pepper plants.

The two potential strains *Streptomyces* sp. (BPSAC34) and *Leifsonia xyli* (BPSAC24) were identified by Passari et al. (2015) with excellent plant growth promotion and when tested *in vivo* showed improvement in a range of growth parameters of chilli (*Capsicum annuum* L.) under greenhouse conditions.

Actinomycetes isolated from the rhizosphere of black pepper and from vermicompost by Bhai et al. (2016) were tested for their antagonistic effect against the causal agents of foot rot and slow decline diseases of black pepper *viz. Phytophthora capsici* and *Radopholus similis*. Based on *in vitro* evaluations of the isolates, four were shortlisted (IISR Act2, IISR Act5, IISR Act6, and IISR Act9) and subjected to *in vivo* evaluation against *Phytophthora* infection by challenge inoculation as well as greenhouse evaluation for growth promotion in black pepper. The results showed that consortia were more effective than individual isolates. Consortia holding IISR Act5+IISR Act9 were found highly efficient in enhancing all the growth parameters followed by IISR Act2+ IISR Act9 and IISR Act2 + IISR Act5.

A greenhouse investigation was carried out with three endophytic actinomycetes; *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis* for their potential to promote plant growth and to protect cucumber from the pathogen *Pythium aphanidermatum* causing damping-off, crown and root rot. It can be used in the nutrient poor soils for crop production as it has good potential to perform as plant growth promoter. As a mechanism of plant growth promotion, these organisms were found producing plant growth regulators i.e., auxins indole-3-acetic acid (IAA) and indole-3-pyruvic acid (IPYA), gibberellic acid (GA3) and cytokinins isopentenyl adenine (iPa) and isopentenyl adenosine (iPA). These three endophytic isolates were screened on the basis of their ability to produce β-1,3, β-1,4 and β-1,6-glucanases to antagonize *P. aphanidermatum*. These endophytes were found to produce glucanase, especially in a consortia treatment, which can be used in place of metalaxyl, a fungicide recommended for pythium diseases in the area. Consortium of

these three was proven better for plant growth promotion and biocontrol as compared to the respective individuals (ElTarabily et al., 2009).

### 2.4.2 Field studies

Thampi and Bhai (2017) conducted surveys with an objective to identify actinomycete strains with growth promotion capability and biocontrol potential from black pepper growing tracts of Kerala and Karnataka for exploiting its antagonistic potential against major pathogens of black pepper as well as for growth promotion. During *in planta* evaluation, the four treatments showed better agronomic performance of black pepper (*Piper nigrum* L.) in terms of growth parameters such as, height of the plant, fresh dry root and shoot biomass, number of nodes and laterals. Out of three isolates, IISRBPAct1 showed significant increase in fresh shoot weight, shoot height and number of nodes. Another peculiar character observed for IISRBPAct1 was the early formation of laterals *i.e.* the fruit bearing branches of black pepper wines, which was significantly high as compared to control plants. However, a well-developed and extensive root system was observed in treatment with IISRBPAct 42 and therefore maximum increase in fresh and dry root biomass was observed in this treatment. The three potential isolates, *Streptomyces* spp., *i.e*. IISRBP Act 1, IISRBP Act 25 and IISRBPAct 42, were very effective in controlling the pathogens under *in vitro* and *in planta* conditions.

## 2.5 COMMERCIAL FORMULATIONS

For the application of growth promoting beneficial actinomycetes in fields and greenhouses, it is mandatory to produce them in large quantities. Development of suitable formulation for the efficient and effective application is the need for the hour. The success of preparation and delivery of any bio formulation depends on the type of organism and site of application (Sabaratnam and Traquair, 2002).

Stevenson (1956) described a medium for delivering *Streptomyces* sp. The medium contained 5 per cent (w/v) sago hampas in soil and a moisture content of 40 per cent. The usage of wheat bran as a carrier material for application in the soil was given by El-Tarabily et al. (2000). Some studies have also been conducted for the development of powder formulation of *S. griseoviridis*, Strain K61 (Mycostop R) for

the management of *Ceratocystis radicicola* in date palm (Suleman et al., 2002) and *Fusarium oxysporum* f. sp. *lycopersici* and *Verticillium dahlia*e in tomato (Minuto et al., 2006). Beausejour et al. (2003) and Jobin et al. (2005) developed chitosan and chitosan-polyphosphate bead formulations of *Streptomyces melanosporofasciens* strain EF-76 respectively and this formulation improved the protection against scab of potato caused by *S. scabies. Streptomyces pseudovenezuelae* strain 13.4.2 was formulated in animal bone charcoal and this showed effectiveness in managing *F. oxysporum* f. sp. *radicis-lycoperisci* (Postma et al., 2013). ActinovateR is another powder formulation of *S. lydicus* strain WYEC108 for the control of *Phytophthora ramorum* (Elliott et al., 2009).

# 3. *Materials & Methods*

# 3. MATERIALS AND METHODS

The present study entitled "Plant growth promoting actinobacteria from rhizosphere soils of black pepper in Wayanad" comprising both laboratory and *in planta* experiments were conducted in Department of Agricultural Microbiology, College of Agriculture, Vellanikkara, during 2018- 2021. Details of materials used and the methods followed are presented below.

## 3.1. MATERIALS USED

### 3.1.1. Chemicals, glassware and plasticware

The chemicals used for the study were Analytical Grade (AR) and procured from the agencies like HIMEDIA, Merck India Ltd. and Sisco Research Laboratory (SRL). Molecular biology reagents and buffers needed for the experiments were purchased from HIMEDIA (Mumbai) and Sigma-Aldrich India Ltd. (Bangalore). Glassware used in the study were purchased from Borosil Ltd, Mumbai and plasticware from Tarson India Ltd. (Kolkata). Kenknight's Agar (Ramasamy et al., 2017), actinomycetes isolation agar (Selvamohan et al., 2016) and starch casein agar (Panigrahi et al., 2011) were used for isolation of actinobacteria. The composition of media is given in Appendix 1.

### 3.1.2. Equipment and machinery

The equipment items needed for the study were available at the Department of Agricultural Microbiology, College of Agriculture, Vellanikkara. Culture media and glassware were sterilized in an autoclave (Equitron-SLEFA of Eutech Instruments, Mumbai). pH meter (Eutech pH Tutor, Singapore) was used for checking the pH of the culture media. Laminar air flow chamber (Rotek Instruments, Vengola, Kerala), was used for inoculation of microorganisms under aseptic conditions. The morphology of microorganisms was visualized under compound binocular microscope (Leica-DM500). Pure cultures of microbes were maintained in glycerol, in ultra-low temperature deep freezer (Haier DW-86L90, Haier International Co. Ltd., China). *In vitro* DNA amplification was completed in Eppendorf Master cycler (Eppendorf,

Germany). Centrifugation was carried out in Table top high speed refrigerated centrifuge (Eppendorf-5804R, Eppendorf, Germany). Visualization of DNA on agarose gel using UV transilluminator (UVP-Benchtop Transilluminator from UVP, USA).

## 3.2. METHODOLOGY

### 3.2.1. Collection of rhizosphere soil samples

Rhizosphere soil samples were collected from five different locations of flood affected and non- flood affected black pepper growing areas of Wayanad district. The soil samples were collected using quartering technique. Global Positioning System (GPS) reading of the locations including latitude and altitude were recorded. About 100 g soil from each location was collected in polythene bag, labelled, sealed and stored in refrigerator till further studies.

### 3.2.2. Isolation of actinobacteria

Isolation and enumeration of actinobacteria from rhizosphere soil samples was carried out by serial dilution and plating technique (Johnson and Curl, 1972).

Ten gram of soil sample was mixed in 90 ml sterile water blank and the contents were mixed thoroughly by shaking for five minutes. One ml of aliquot was transferred to 9 ml water blank and serially diluted to a final dilution of $10^{-3}$ and $10^{-4}$ for non- flood affected areas and $10^{-1}$ for flood affected areas. Then 1 ml of suspension from respective dilutions was transferred aseptically to Petri dishes. Twenty ml aliquots of molten and cooled agar of actinomycetes isolation agar, Kenknight & Munaier's agar and starch casein agar were poured into Petri plates. The plates were rotated clockwise and anti-clockwise directions and the medium allowed to solidify. Three replicates were used for each medium and each dilution. Plates were incubated at $28^0$C for 5-12 days. Number of colonies on the respective media was counted and population expressed as colony forming units per gram of soil (cfu $g^{-1}$).

### 3.2.3. Purification and maintenance of isolates

The single colonies of actinobacteria obtained were repeatedly streaked on a Kenknight & Munaier's agar and single discreet colonies were obtained. Pure cultures obtained by repeated subcultures were maintained on agar slants and as glycerol stock at -80$^{o}$C in deep freezer.

### 3.2.4. Characterization of isolates

#### 3.2.4.1. Morphological and cultural characterization

The actinobacteria were grown on Kenknight & Munaier's agar media at 28$^{o}$C for 7 days. The characters such as colony appearance, type of aerial hyphae were observed. The colour of spore masses and diffusible pigment production were visualized. Gram staining (Hucker and Conn, 1923) was carried out to obtain Gram reaction. Spore chain morphology of the isolates were observed under microscope.

#### 3.2.4.2. Biochemical characterization

##### 3.2.4.2.1. Oxidase test

The isolates were smeared on oxidase discs (Hi-media DD018-1VL). Appearance of violet colour within 5-10 seconds at 25-30$^{o}$ C indicated positive reaction. A colour change after 60 seconds or no colour change was considered as negative reaction (Biswas et al., 2020).

##### 3.2.4.2.2. Catalase test

Smear of the isolates was prepared on clean glass slides and a drop of hydrogen peroxide (3%) was added to it. Cultures which immediately showed effervescence were treated as positive (Taylor and Achanzar, 1972).

##### 3.2.4.2.3. Utilization of sugars

Fermentation broth (containing glucose, fructose and sucrose) was prepared in test tubes. Durham's tubes were placed in an inverted position in the broth. The isolates

were inoculated in broth and uninoculated control was also maintained. Three replications were maintained and incubated for $37^0$C for 5 days. A change in colour from red to yellow indicated fermentation and appearance of bubbles indicated gas production (Cowan, 1974).

### 3.2.5. *In vitro* screening for plant growth promoting (PGP) activities

All the isolates were screened for plant growth promoting activities like indole acetic acid (IAA) production, nitrogen fixation, phosphate solubilization, potash solubilization and zinc solubilization under *in vitro* conditions.

#### 3.2.5.1. Screening of actinobacterial isolates for IAA production

The actinobacterial isolates were screened for the production of IAA (Ahmed et al., 2008). All isolates were inoculated in sterile Luria-Bertani supplemented with tryptophan at the rate of 1 mg $ml^{-1}$. The tubes were incubated for 7 days in the dark. The cultures were centrifuged at 3000 rpm for 30 minutes and the supernatant was collected. Four ml Salkowski reagent was added to the supernatant. Development of pink colour indicated positive reaction for IAA production.

#### 3.2.5.2. Estimation of IAA production by isolates

The isolates were inoculated in sterile Luria-Bertani supplemented with tryptophan at the rate of 1 mg $ml^{-1}$ (Ahmed et al., 2008). The tubes were incubated for 7 days in the dark. The cultures were centrifuged at 3000 rpm for 30 minutes and the supernatant was collected. 4 ml Salkowski reagent was added to the supernatant. The pink colour developed was used for measuring the optical density at 530 nm using a spectrophotometer. The OD values were plotted on a standard graph to obtain the quantity of IAA produced by the isolates and expressed as µg $ml^{-1}$ of broth.

#### 3.2.5.3. Screening of actinobacterial isolates for nitrogen fixation

Actinobacterial isolates were streaked on N-free Jensen's agar. The inoculated plates were then incubated at 28 ± 2 $^{o}$C for six days. The isolates which were able to grow on N free media were considered as nitrogen fixers. Based on the growth on the media, nitrogen fixers were rated as excellent (++++), good (+++), moderate (++), poor (+) and no growth (-).

### 3.2.5.4. Estimation of nitrogen fixation

Nitrogen fixation by the thirty-five isolates was quantified by micro-Kjeldahl method (Jackson, 1973 and Bremner, 1960). A loopful of culture was inoculated in 5 ml Jensen's broth in a glass tube and incubated for 6 days. 1 ml of this culture was inoculated in 50 ml of Jensen's broth in 250 ml conical flasks and three replicates were maintained for each isolate.

After 15 days of incubation, the cultures were homogenized by shaking. Ten ml of the homogenized culture was drawn and mixed with 10 ml concentrated $H_2SO_4$ and 1g of digestion mixture (copper sulphate: selenium in the ratio 20:1) in the ratio 10:1. The mixture was kept for digestion overnight at room temperature and then in a block digester for 2 hours at 300 $^{0}$C till it became clear. The clear digest was cooled and transferred to Kjeldahl's distillation unit. Ten ml of NaOH (40%) was added and condensed $NH_3$ was trapped in 10 ml boric acid indicator mixture (4% boric acid solution in hot water). Four ml of mixed indicator solution (0.2% bromocresol green + 0.2% methyl red in alcohol with 5:1 ratio) was added to 1000 ml of 4% boric acid solution. The colour changed from reddish pink to bluish green as the $NH_3$ was trapped in indicator. After this process was over, it was titrated against 0.01N HCl until the solution turned back to reddish pink. A blank was also used for titration. Total nitrogen content of the cultures was determined and the results were expressed as mg N fixed per gram of carbon source utilized.

$$\text{mg of N/ g of C source} = \frac{\text{TV-BV x N x 0.014 x 1000}}{\text{Y}}$$

where,

TV = Titre value

BV = Blank value

N = Normality of $H_2SO_4$

Y = Weight of carbon source

### 3.2.5.5. Screening of actinobacterial isolates for phosphate solubilization

All the actinobacterial isolates were screened for phosphate solubilization on Pikovskaya's agar (Nguyen et al., 1992). The isolates were inoculated in Pikovskaya's agar containing tricalcium phosphate and incubated for five to seven days at 28 ± 2°C. The halo zone and colony diameter were measured after seven days of incubation. The ability to solubilize insoluble phosphate was expressed as per cent solubilization efficiency (SE)

$$\text{Solubilizing efficiency (\% SE)} = \frac{SD}{CD} \times 100$$

SD - Solubilization diameter (mm)

CD - Colony diameter (mm)

### 3.2.5.6. Estimation of phosphate solubilization by actinobacterial isolates

The isolates which showed positive reaction for phosphate solubilization on Pikovskaya's agar containing tricalcium phosphate in the preliminary screening were further subjected to quantification of phosphorus solubilization using phospho-molybdic blue colour method (Olsen et al., 1962). Flasks containing 50 ml Pikovskaya's broth were inoculated with 500 µl of overnight grown culture of each isolate. Pikovskaya's broth without inoculum served as control and all the tubes were incubated for 14 days at 28 ± 2 °C.

At the end of incubation, the cultures were centrifuged at 10,000 rpm for 10 minutes to separate the cells and insoluble phosphate. Five ml of the supernatant was taken in a test tube and the volume was made up to 8.6 ml with distilled water. One ml of ammonium molybdate reagent and 0.4 ml of ANSA reagent were added to the tubes. The contents were mixed thoroughly and allowed to stand for 10 minutes for colour

development. Intensity of the blue colour was read in a spectrophotometer at 660 nm. The amount of available phosphorus present in the broth was calculated by referring to the standard graph of different known concentration of phosphorus using $KH_2PO_4$. The pH of the supernatant was also recorded so as to assess the reduction in pH from the initial value of 7.2 after 14 days of incubation.

### 3.2.5.7. Screening of actinobacterial isolates for potassium solubilization

All the actinobacterial isolates obtained were screened for K solubilization on Aleksandrov's agar (Panhwar et al., 2012). The isolates were uniformly spotted on Aleksandrov's agar plate. Inoculated plates along with uninoculated control were sealed properly and incubated for seven days at 28 ± 2°C. The halo zone and colony diameter were measured after seven days of incubation. The efficiency of solubilization was expressed as per cent solubilization efficiency (SE).

$$\text{Solubilizing efficiency (\% SE)} = \frac{SD}{CD} \times 100$$

SD - Solubilization diameter (mm)

CD - Colony diameter (mm)

### 3.2.5.8. Screening of actinobacterial isolates for zinc solubilization

Actinobacterial isolates obtained were screened for zinc solubilization on mineral salt agar medium, amended with 0.1% of insoluble zinc oxide (Venkatakakrishnan et al., 2004). The isolates were spot-inoculated on the medium. Inoculated plates along with uninoculated control were sealed properly and incubated for 15 days at 28 ± 2°C. The clear zone and colony diameter were measured after 15 days of incubation. The efficiency of solubilization was expressed as per cent solubilization efficiency (SE).

$$\text{Solubilizing efficiency (\% SE)} = \frac{SD}{CD} \times 100$$

SD - Solubilization diameter (mm)

CD - Colony diameter (mm)

### 3.2.6. Molecular characterization

Selected isolates of actinobacteria exhibiting plant growth promoting activities were identified by 16S rRNA gene sequencing.

#### 3.2.6.1. 16S rRNA gene sequence analysis of selected isolates

The unknown isolates were subjected to molecular characterization by 16S rRNA gene sequencing for identification.

#### 3.2.6.2. Amplification of 16S rRNA gene

Amplification of 16S rRNA gene was carried out by colony PCR (Woodman, 2008). A single isolated colony was taken using a microtip and mixed with 10 µl of sterile water. For amplification of 16S rRNA gene, 2 µl of the suspension was used as template. The details about the primers used are given in Table 1.

Polymerase chain reaction was carried out in Eppendorf Master Cycler (Gradient) using 'Emerald Amp GT PCR' PCR master mix. The details of the PCR reaction mixture are given in Table 2. A momentary spin was given to mix and the reaction set in master cycler. The details of master cycler programme are specified in Table 3.

**Table 1. Details of primers used for 16S rRNA gene amplification**

| Primer details | Sequence 5'- 3' | Length in bp |
|---|---|---|
| 16S-RS-F | CAG GCC TAA CAC ATG CAA GTC | 21 |
| 16S-RS-R | GGG CGG GTG TAT ACA AGG C | 19 |

**Table 2. Composition of PCR reaction mixture**

| Component | Per reaction volume required (µl) |
|---|---|
| 2 x Master mix | 5.0 |
| Template | 1.0 |
| Forward primer | 0.25 |
| Reverse primer | 0.25 |
| $dH_2O$ | 4.0 |
| Total | 10.50 |

**Table 3. Details of master cycler programme**

| No. | Step | Temperature ($^0$C) | Time |
|---|---|---|---|
| 1 | Initial denaturation | 95 | 5.00 min |
| 2 | Denaturation | 95 | 30 sec |
| 3 | Annealing | 60 | 40 sec |
| 4 | Primer extension | 72 | 60 sec |
| 5 | Steps 2-4 | 35 cycles | - |
| 6 | Final extension | 72 | 7.00 min |
| 7 | Final hold | 4 | $\alpha$ |

### 3.2.6.3. Agarose gel electrophoresis of PCR products

Evaluation of quality of isolated DNA was carried out by agarose gel electrophoresis (Sambrook et al., 1989). The PCR products were checked on 1.2% agarose gels prepared in 1X TAE buffer containing 0.5 µg $ml^{-1}$ ethidium bromide. The gel casting tray was set up with properly placed comb and agarose was poured into the tray with care and allowed to solidify. The comb was carefully removed to obtain wells

and 1X TAE buffer was poured into the tank. 1 µl of 6X loading dye was mixed with 2 µl of PCR products and was loaded and electrophoresis was performed at 75 V power supply for about 1-2 hours, until the bromophenol blue had migrated to two third length of the gel.

#### 3.2.6.4. Gel documentation

After the separation of DNA bands using electrophoresis, the gels were visualized in a UV transilluminator and the image was taken under UV light using gel documentation system.

#### 3.2.6.5. Purification and sequencing of PCR product

The PCR product was then purified and sequenced at Rajiv Gandhi Centre for Biotechnology, Trivandrum using the primer 16S-RS-F and 16S-RS-R. The sequencing reaction of the PCR product was done in a PCR thermal cycler using the Big dye terminator v3.1. The sequence quality was checked using sequence scanner software V1.

#### 3.2.6.6. Nucleotide sequence analysis

Sequence analysis and nucleotide homology of each isolates were identified through the BLASTn (basic local alignment search tool) programme of NCBI (National Centre for Biotechnology Information) (http://www.ncbi.nlm.nih.gov). The accession sharing maximum homology with the query sequence was used to identify the isolates.

#### 3.2.6.7. Construction of phylogenetic tree

Phylogenetic tree of selected actinobacterial isolates was constructed on the basis of 16S rRNA gene sequencing using the MEGA7 software (Hall, 2013). 16S rRNA gene sequences of the isolates obtained in the study, along with the sequences of four accessions in NCBI database, showing homology with the query sequences were considered for the construction of phylogenetic tree. Bootstrap value of 100 was used for the construction of phylogenetic tree.

### 3.2.7. Evaluation of selected actinobacteria for growth promotion in black pepper cuttings

The three most promising and efficient isolates were evaluated under sterile conditions for their growth promotion efficiency in black pepper. The experiment was conducted during May to October 2020 in the net house of Department of Agricultural Microbiology, Vellanikkara.

Treatment details:

Design : CRD

Replications : 3*

Treatments : 6

Variety : Panniyur 1

$T_1$: Selected actinobacteria-1

$T_2$: Selected actinobacteria -2

$T_3$: Selected actinobacteria -3

$T_4$: PGPR Mix-1

$T_5$: Organic POP recommendation of KAU (2017)

$T_6$: Absolute control

* Each replication contains four polybags, each with four cutting

#### 3.2.7.1. Preparation of potting mixture and planting

The potting mixture was prepared with sand: soil: farmyard manure (1:1:1) and it was sterilized by adding 5% formaldehyde solution. The potting mixture was covered with a polythene film to retain the moisture content. After 15 days, the mixture was raked thoroughly and left open for 7 days. The sterile potting mixture was filled in polythene bags (16 x 14 inches and 150 gauge thickness). One month old rooted black pepper cuttings of Panniyur-1, at two leaf stage, having uniform growth were selected

for planting. These rooted cuttings were obtained from Model Nursery on Spices, College of Agriculture, Vellanikkara.

### 3.2.7.2. Enumeration of actinobacteria in sterile potting mixture

Population of actinobacteria in potting mixture was enumerated by serial dilution and plating technique using Kenknight's agar media.

### 3.2.7.3. Preparation and application of selected actinobacterial isolates

Talc-based formulation of three actinobacterial isolates were prepared by inoculating loopful of individual colonies from agar plates in 100 ml of Kenknight's broth and incubated at 28$^{o}$C for 7 days. After incubation, the inocula were uniformly shaken and mixed with talc in the ratio of 1:3. Then serial dilution and plating was carried out to enumerate actinobacteria in the formulation ($10^{8}$cfu $g^{-1}$). Soil was inoculated with the microbial suspension at the time of planting and 45 days after planting.

### 3.2.7.4. Biometric observations

Observations on vine length, number of leaves and internode length were recorded at monthly intervals up to five months after planting. Fresh weight and dry weight of plant was also recorded at the end of five months. Root parameters like root volume, fresh and dry weight of roots were also recorded at the end of five months after planting.

#### 3.2.7.4.1. Vine length

The distance from the base of the plant at the soil level to the tip of vine was recorded at monthly interval up to 5 MAP and expressed in cm.

#### 3.2.7.4.2. Number of leaves

Total number of leaves on each plant were counted at monthly interval for 5 MAP and mean number of leaves was recorded.

**3.2.7.4.3. Internode length**

Internode length on each plant was measured at monthly interval for 5 MAP and mean internode length was taken.

**3.2.7.4.4. Fresh weight and dry weight of roots**

Plants were uprooted, root system separated, cleaned and the fresh weight was recorded. Dry weight was also recorded after drying in an oven at 60 ± 5 $^{o}$C for 72 hours till constant weight (g) was reached.

**3.2.7.4.5. Fresh weight and dry weight of plants**

Plants were uprooted, roots were cleaned and the fresh weight was taken. Dry weight was also recorded after drying in an oven at 60 ± 5 $^{o}$C for 72 hours till constant weight (g) was reached.

**3.2.7.4.6. Root volume**

Plants were uprooted, roots separated and cleaned. Then root volume was measured by the amount of water displaced, while immersing the roots in a container.

Root volume = Volume of the water after submerging roots into the cylinder - volume of the water before submerging the roots

**3.2.8. Statistical analysis**

Analysis of variance was done on the data collected by using the statistical package WASP 2.0. (Gomez and Gomez, 1984).

# 4. Results

# 4. RESULTS

The results of the study entitled "Plant growth promoting actinobacteria from rhizosphere soils of black pepper in Wayanad" conducted during the period 2018-2020 at Department of Agricultural Microbiology, College of Horticulture, Vellanikkara are presented in this chapter.

## 4.1. COLLECTION OF RHIZOSPHERE SOILS OF BLACK PEPPER FROM WAYANAD DISTRICT

Ten rhizosphere soil samples were collected from black pepper gardens in different locations of Wayanad district (Plate 1). These 10 samples comprised five each from non-flood affected and flood affected black pepper areas of Wayanad (Fig 1). The samples were collected during the month of January and February, 2019. Details of soil samples collected along with their geographical positions are given in Table 4.

## 4.2. ISOLATION OF ACTINOBACTERIA

Actinobacteria were isolated using three different media *viz.* Actinomycetes Isolation Agar, Kenknight and Munaier's agar and Starch Casein Agar (Plate 2), following serial dilution plate method. Typical actinobacteria colonies could be distinguished from other bacterial or fungal colonies by their leathery and hard texture.

Among different soil samples analysed, Puttad (Ptd) recorded significantly superior actinobacterial population on all the three media, with a population of 12.00 $x10^4$ cfu $g^{-1}$ on starch casein agar, $6.67x10^4$ cfu $g^{-1}$ on Actinomycetes Isolation Agar and $7.00x10^4$ cfu $g^{-1}$ on Kenknight and Munaier's agar. Ambalavayal soil also recorded significantly higher population of actinobacteria ($7.00x10^4$ cfu $g^{-1}$) on Starch Casein Agar. Lowest population was observed in Pathiri (Ptr) on Kenknight and Munaier's agar and Actinomycetes Isolation Agar (Table 5). No actinobacterial colonies were detected in any of the flood affected soil samples on any medium, even at a dilution of $10^{-1}$ (Table 6), except in Meppadi soil, which recorded a population of $0.3x10^1$ cfu $g^{-1}$.

Among the different media, Starch Casein recorded higher population in all the non-flooded locations, except Pulpally (Table 5).

The colonies were purified by repeated sub-culturing on Kenknight and Munaier's agar medium (Plate 3). A total of 35 actinobacteria (34 from non-flooded areas and one from flood affected area) with different morphological characters were selected for further studies. All the isolates were maintained on Kenknight and Munaier's agar slants in refrigerator and glycerol stock as per standard procedure.

## 4.3 CHARACTERIZATION OF ISOLATES

### 4.3.1. Morphological and cultural characterization

Cultural characters of all the 35 isolates were assessed by growing them on Kenknight Munaier's agar for 6 to 8 days. The same isolates on different media showed wide variation in colony morphology. The isolate Vlt-K produced yellow pigment on Kenknight and Munaier's Agar, whereas the same isolate did not produce any pigment on Starch Casein Agar. The aerial mycelium of Amb-B was white on Starch Casein Agar, whereas on Kenknight and Munaier's agar was dark brown in colour (Plate 4). The colour of aerial mycelium varied from dark brown to light brown, white, purple or green. Colonies were circular or irregular, with the exception of Amb-A and Plt-A, which were filamentous, and Plp-A and Plp-C which were rhizoid. The elevation, form and margin of colonies varied among the isolates. Two isolates (Vlt-K and Plp-B) produced yellow water-soluble pigment and Vlt-I produced water-insoluble purple pigment. The spore chain morphology varied among genera and 14 isolates produced rectiflexibile type of sporulating aerial hypae *ie*. straight or flexuous spore chains, partly in fascicles (Table 6 and Plate 5). Spira type, short chain and closed spira of sporulating aerial hyphae were observed in six isolates each. Single spore, sporangia formation and filamentous type of aerial hyphae were observed in one isolate each. All the 35 isolates were Gram positive in reaction (Plate 5).

### 4.3.2. Biochemical characterization

Among 35 isolates, 26 isolates were positive to catalase test (Plate 6 and Table 7) and nine isolates (Ptd-B, Amb-A, Amb-B, Amb-C, Amb-G, Vlt-B, Vlt-E, Ptr-D and Ptr-E) negative. Thirteen isolates *viz.* Ptd-E, Amb-A, Amb-B, Amb-F, Amb-H, Vlt-B, Vlt-D, Vlt-G, Ptr-C, Ptr-E, Ptr-F, Plp-A and Mpd-A produced purple colour on the oxidase disc, indicating positive reaction to oxidase test (Plate 7 and Table 7). Three

isolates (Ptd-A, Vlt-B and Vlt-G) produced a colour change of the broth from red to yellow, without any gas formation, which indicated that they were positive to glucose fermentation test. None of the isolates could utilize either fructose or sucrose (Plate 8 and Table 7).

**Table 4. Locations of soil sample collection**

| Location | Code | Latitude | Longitude |
|---|---|---|---|
| **Non-flood affected** | | | |
| Puttad | Ptd | $11.57843^0$ | $76.17174^0$ |
| Amb-Alavayal | Amb | $11.61970^0$ | $76.1059^0$ |
| Valat | Vlt | $11.79405^0$ | $75.90547^0$ |
| Pathiri | Ptr | $11.8434^0$ | $76.17174^0$ |
| Pulpally | Plp | $11.79213^0$ | $76.16624^0$ |
| **Flood affected** | | | |
| Vellamunda | Vlm | $11.73359^0$ | $75.93748^0$ |
| Vythiri | Vth | $11.55097^0$ | $76.04021^0$ |
| Panamaram | Pnm | $11.73779^0$ | $76.07398^0$ |
| Adikkolly | Adk | $11.78144^0$ | $76.18896^0$ |
| Meppadi | Mpd | $11.55514^0$ | $76.13529^0$ |

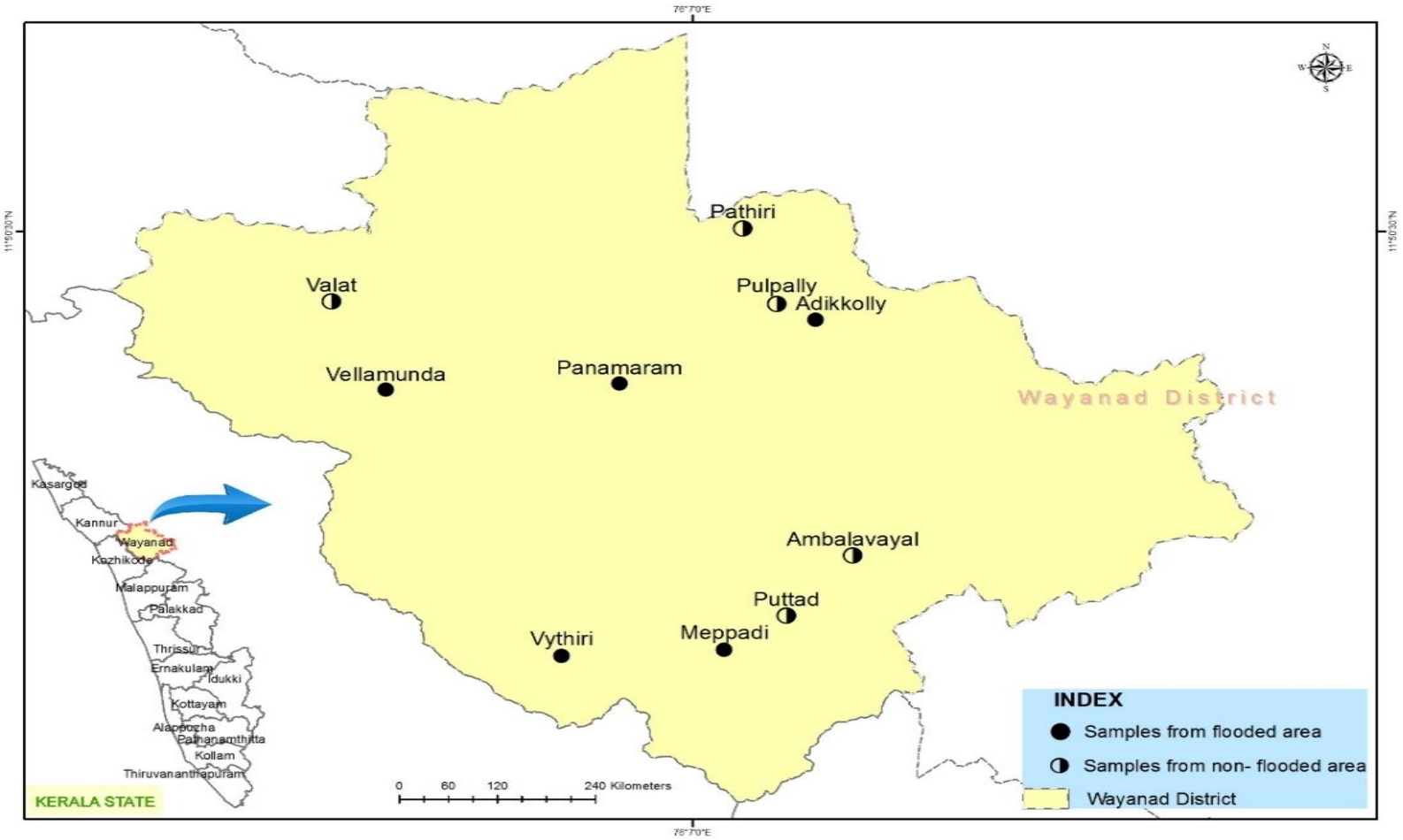



**Fig 1. Locations of soil samples collected from Wayanad districts**

**Panamaram**

**Meppadi**

**Puttad**

**Ambalavayal**

**Plate 1. Black pepper gardens in Wayanad selected for rhizosphere soil collection**

**Table 5. Enumeration of actinobacteria from non-flood and flood affected rhizosphere soils of black pepper in Wayanad**

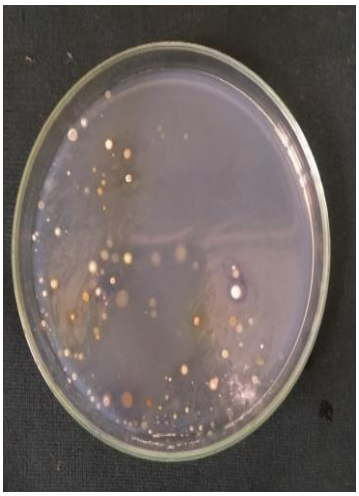
| 1. Non flooded areas | | | | |
|---|---|---|---|---|
| Location | Code | Population of actinobacteria (x $10^4$ cfu $g^{-1}$) | | |
| | | Kenknight & Munaier's agar | Actinomycetes Isolation Agar | Starch Casein Agar |
| Puttad | Ptd | 7.00 (0.84)$^{a}$ | 6.67 (0.82)$^{a}$ | 12.00 (1.08)$^{a}$ |
| Amb-Alavayal | Amb | 5.00 (0.59)$^{b}$ | 3.00 (0.48)$^{b}$ | 7.00 (0.84)$^{a}$ |
| Valat | Vlt | 3.00 (0.48)$^{b}$ | 2.00 (0.30)$^{c}$ | 4.33 (0.63)$^{c}$ |
| Pathiri | Ptr | 2.00 (0.30)$^{c}$ | 1.00 (0.00)$^{d}$ | 2.67 (0.42)$^{d}$ |
| Pulpally | Plp | 3.00 (0.46)$^{bc}$ | 3.00 (0.48)$^{b}$ | 2.00 (0.30)$^{e}$ |

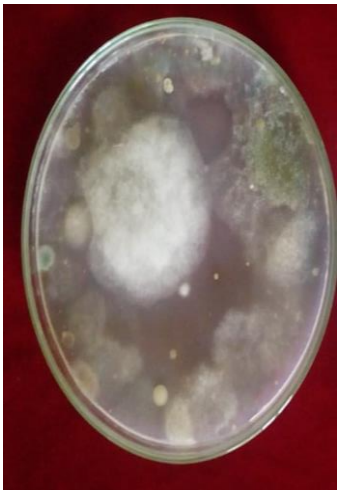
| 2. Flooded areas | | | | |
|---|---|---|---|---|
| Location | Code | Population of actinobacteria (x $10^1$) cfu $g^{-1}$ | | |
| | | Kenknight & Munaier's agar | Actinomycetes Isolation Agar | Starch Casein Agar |
| Vellamunda | Vlm | 0.00 | 0.00 | 0.00 |
| Vythiri | Vth | 0.00 | 0.00 | 0.00 |
| Panamaram | Pnm | 0.00 | 0.00 | 0.00 |
| Adikkolly | Adk | 0.00 | 0.00 | 0.00 |
| Meppadi | Mpd | 0.00 | 0.00 | 0.30 |

Log transformed values in parantheses

**Table 6. Morphological and cultural characterization of isolates**

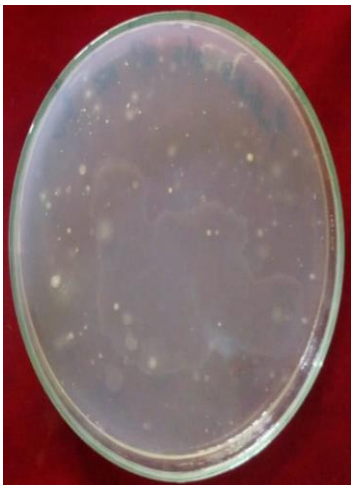
| Isolates | Colony colour | Elevation | Form | Margin | Gram staining | Pigment produced | Spore chain morphology |
|---|---|---|---|---|---|---|---|
| Ptd-A | Dark brown | Raised | Circular | Curled | + | - | Rectiflexibiles |
| Ptd-B | Off white | Umbonate | Circular | Undulate | + | - | Spira |
| Ptd-C | Light brown | Raised | Irregular | Undulate | + | - | Rectiflexibiles |
| Ptd-D | Brown | Umbonate | Circular | Entire | + | - | Closed spira |
| Ptd-E | Brown with white outer | Raised | Irregular | Entire | + | - | Filamentous |
| Amb-A | White | Umbonate | Circular | Filiform | + | - | Rectiflexibiles |
| Amb-B | Brown | Raised | Circular | Filiform | + | - | Rectiflexibiles |
| Amb-C | Light brown | Raised | Circular | Entire | + | - | Closed spira |
| Amb-D | Off white | Flat | Irregular | Undulate | + | - | Rectiflexibiles |
| Amb-E | White (slow) | Flat | Irregular | Filiform | + | - | Spira |
| Amb-F | Green | Raised | Circular | Filiform | + | - | Spira |
| Amb-G | White dusty | Raised | Filamentous | Filiform | + | - | Spira |
| Amb-H | Rose (light) | Umbonate | Circular | Filiform | + | - | Short chain |
| Vlt-A | Fine growth no color | Flat | Irregular | Undulate | + | - | Rectiflexibiles |
| Vlt-B | White with brown centre | Raised | Circular | Filiform | + | - | Rectiflexibiles |
| Vlt-C | Off white | Raised | Irregular | Undulate | + | - | Rectiflexibiles |
| Vlt-D | Creamy brown | Flat | Irregular | Undulate | + | - | Rectiflexibiles |

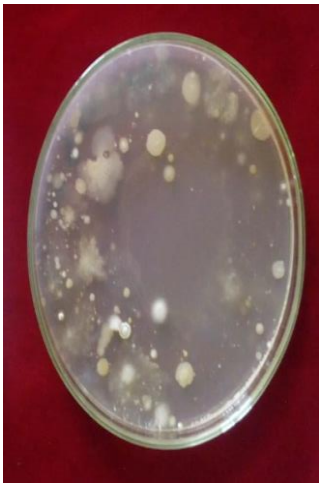




**Kenknight's agar** **Actinomycetes isolation agar** **Starch casein agar**

**Non-flood affected area (Puttad)**





**Kenknight's agar** **Actinomycetes isolation agar** **Starch casein agar**

**Flood affected area (Meppadi)**

**Plate 2. Rhizosphere actinobacteria isolated on different media**

| Isolates | Colony colour | Elevation | Form | Margin | Gram staining | Pigment produced | Spore chain morphology |
|---|---|---|---|---|---|---|---|
| Vlt-E | Light brown | Umbonate | Irregular | Entire | + | - | Spira |
| Vlt-F | White (ring like) | Flat | Circular | Undulate | + | - | Short chain |
| Vlt-G | Light brown | Flat | Filamentous | Curled | + | - | Rectiflexibiles |
| Vlt-H | White | Umbonate | Circular | Entire | + | - | Sporangia |
| Vlt-I | Off white | Flat | Circular | Undulate | + | Purple | Short chain |
| Vlt-J | White with brown centre | Umbonate | Circular | Entire | + | - | Single spore |
| Vlt-K | Light brown | Umbonate | Circular | Curled | + | yellow | Rectiflexibiles |
| Vlt-L | White | Umbonate | Circular | Curled | + | - | Rectiflexibiles |
| Ptr-A | Creamy brown | Umbonate | Circular | Entire | + | - | Closed spira |
| Ptr-B | Creamy | Raised | Irregular | Curled | + | - | Rectiflexibiles |
| Ptr-C | Light brown | Umbonate | Circular | Filiform | + | - | Closed spira |
| Ptr-D | Dark brown | Umbonate | Circular | Curled | + | - | Short chain |
| Ptr-E | Off white with brown | Umbonate | Circular | Filiform | + | - | Spira |
| Ptr-F | White | Umbonate | Circular | Undulate | + | - | Closed spira |
| Plp-A | Ashy green | Slightly raised | Rhizoid | Undulate | + | - | Closed spira |
| Plp-B | Brown | Umbonate | Irregular | Undulate | + | yellow | Short chain |
| Plp-C | Brown | Umbonate | Rhizoid | Undulate | + | - | Rectiflexibiles |
| Mpd-A | Light brown | Slightly raised | Circular | Undulate | + | - | Short chain |

**Table 7. Characterization of isolates based on biochemical tests**

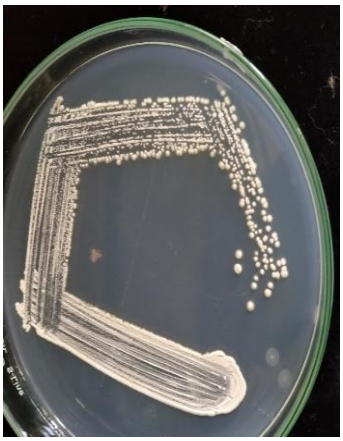
| Isolates | Catalase | Oxidase | Utilization of sugars | | |
|---|---|---|---|---|---|
| | | | Glucose | Fructose | Sucrose |
| Ptd-A | + | - | + | - | - |
| Ptd-B | - | - | - | - | - |
| Ptd-C | + | - | - | - | - |
| Ptd-D | + | - | - | - | - |
| Ptd-E | + | + | - | - | - |
| Amb-A | - | + | - | - | - |
| Amb-B | - | + | - | - | - |
| Amb-C | - | - | - | - | - |
| Amb-D | + | - | - | - | - |
| Amb-E | + | - | - | - | - |
| Amb-F | + | + | - | - | - |
| Amb-G | - | - | - | - | - |

**Plp-C**

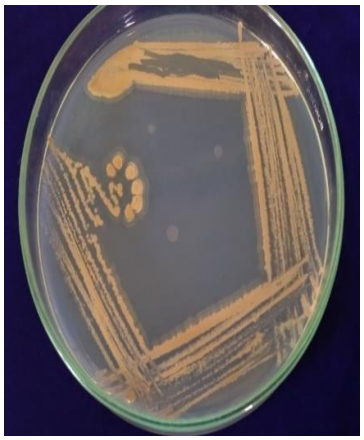
**Vlt-I**

**Vlt-K**

**Plp-B**



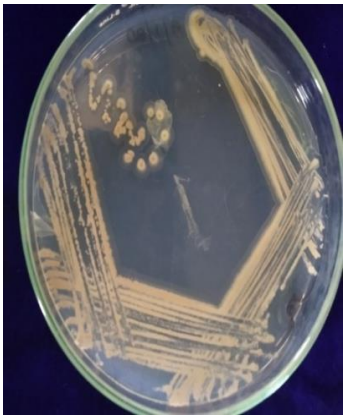
**Amb-C**



**Ptd-E**

**Plp-A**

**Ptr-A**



**Ptd-A**

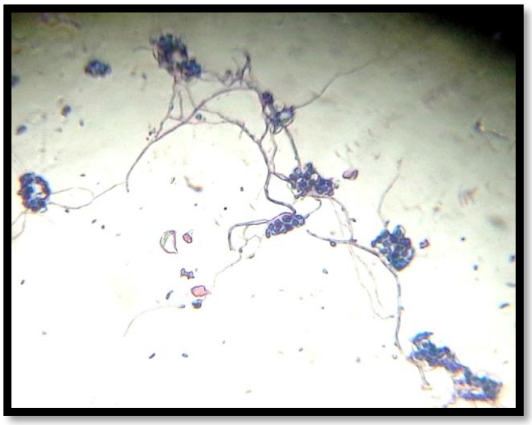
**Plate 3. Pure actinobacteria isolates on Kenknight's & Munaier's Agar media**

**Starch Casein Agar** **Kenknight & Munaier's Agar**

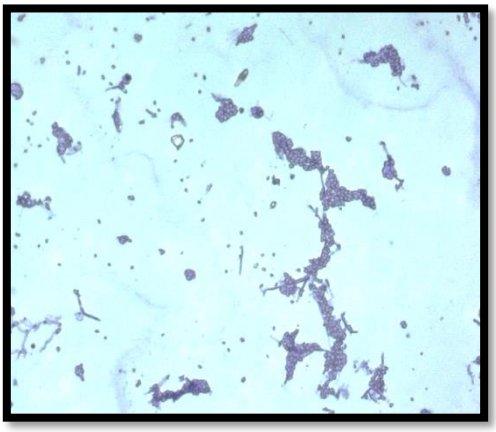
**Vlt-K**

**Amb-B**

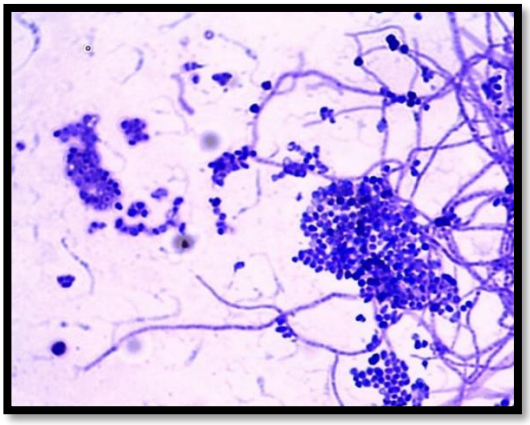
**Plate 4. Colony morphology of actinobacterial isolates on different media**



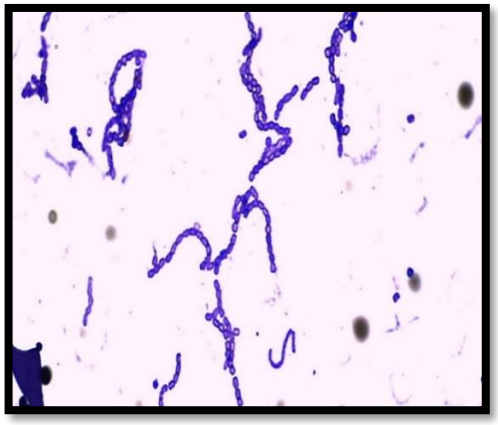
**Ptr-A**



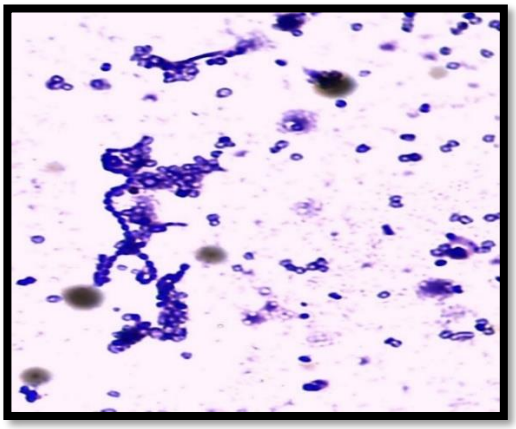
**Ptd-A**



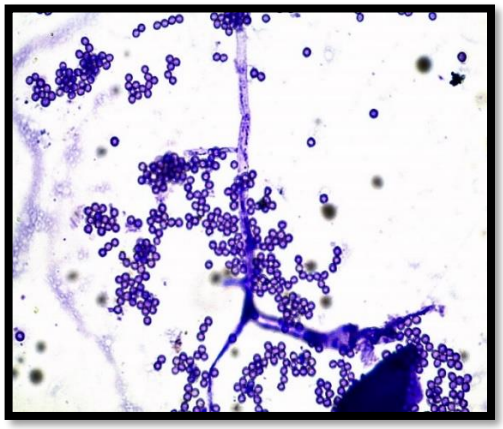
**Vlt-E**



**Amb-H**



**Ptr-B**



**Vlt-F**

**Plate 5. Microscopy view of isolates under 40X magnification**

| Isolates | Catalase | Oxidase | Utilization of sugars | | |
|---|---|---|---|---|---|
| | | | Glucose | Fructose | Sucrose |
| Amb-H | + | + | - | - | - |
| Plt-A | + | - | - | - | - |
| Vlt-B | - | + | + | - | - |
| Vlt-C | + | - | - | - | - |
| Vlt-D | + | + | - | - | - |
| Vlt-E | - | - | - | - | - |
| Vlt-F | + | - | - | - | - |
| Vlt-G | + | + | + | - | - |
| Vlt-H | + | - | - | - | - |
| Vlt-I | + | - | - | - | - |
| Vlt-J | + | - | - | - | - |
| Vlt-K | + | - | - | - | - |
| Vlt-L | + | - | - | - | - |
| Ptr-A | + | - | - | - | - |

| Isolates | catalase | oxidase | Utilization of sugars | | |
|---|---|---|---|---|---|
| | | | Glucose | Fructose | Sucrose |
| Ptr-B | + | - | - | - | - |
| Ptr-C | + | + | - | - | - |
| Ptr-D | - | - | - | - | - |
| Ptr-E | - | + | - | - | - |
| Ptr-F | + | + | - | - | - |
| Plp-A | + | + | - | - | - |
| Plp-B | + | - | - | - | - |
| Plp-C | + | - | - | - | - |
| Mpd-A | + | + | - | - | - |

**Plate 6. Catalase test**

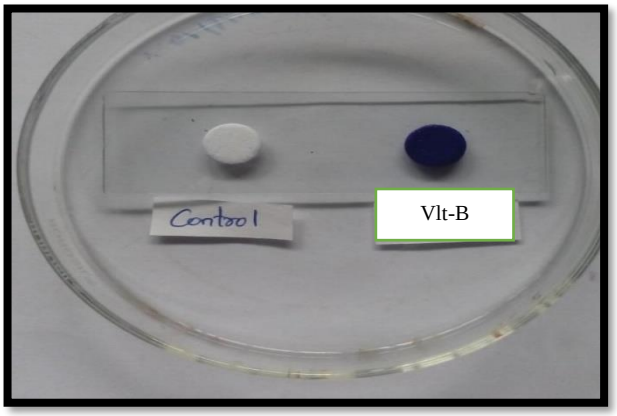
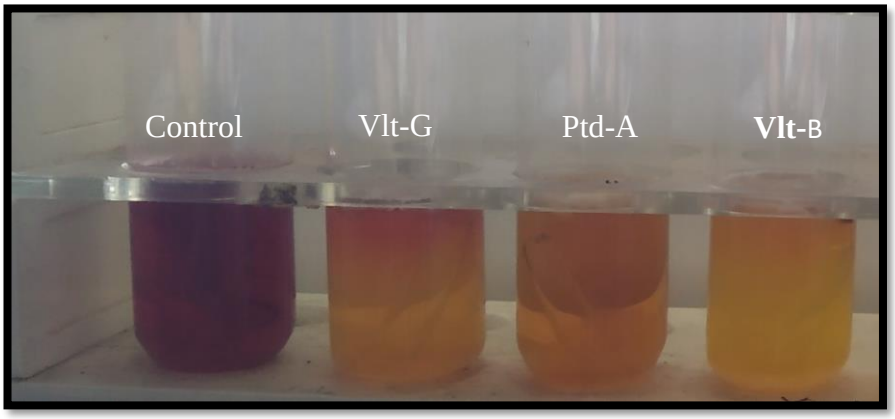
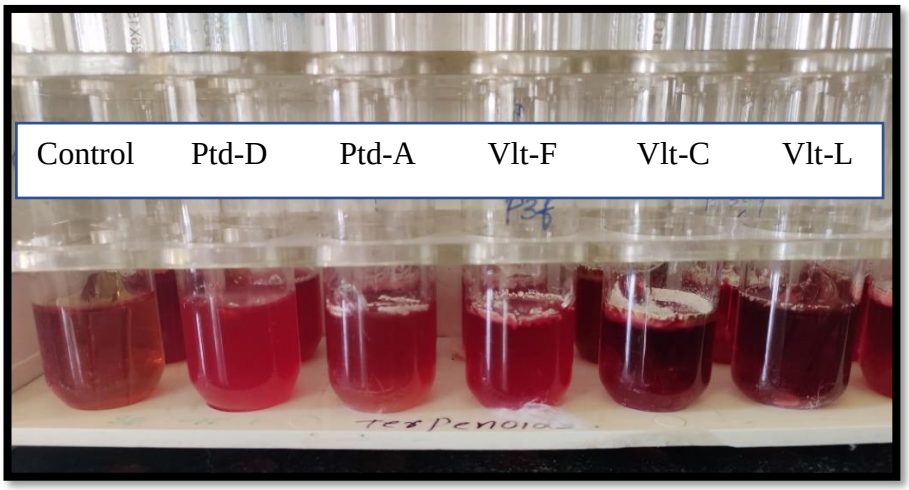


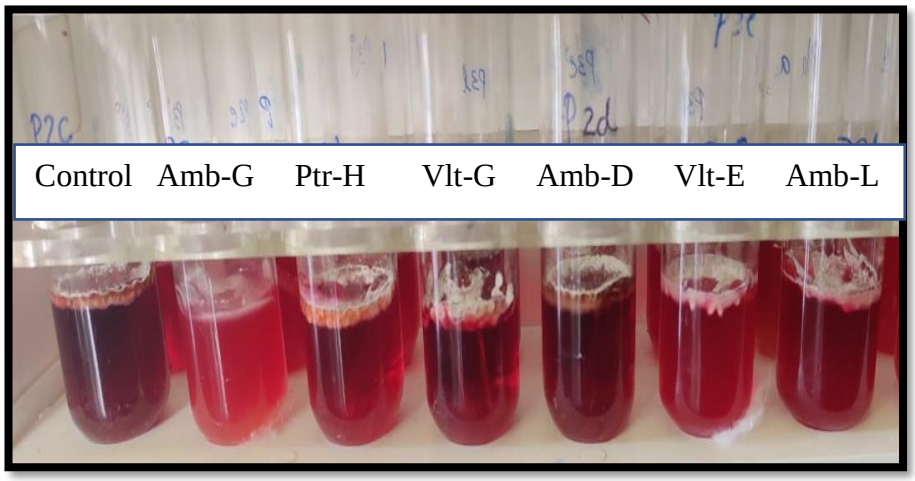

**Plate 7. Oxidase test**




**A. Glucose test**




**B. Fructose test**




**C. Sucrose test**

**Plate 8. Sugar fermentation tests**

## 4.4. *In vitro* SCREENING OF ACTINOBACTERIAL ISOLATES FOR PLANT GROWTH PROMOTING (PGP) ACTIVITIES

Thirty-five isolates of actinobacteria obtained from different locations of Wayanad were screened for various plant growth promoting activities including the production of indole acetic acid (IAA), fixation of atmospheric nitrogen, solubilisation of phosphate, potassium and zinc, under *in vitro* conditions.

### 4.4.1. Screening of actinobacterial isolates for indole acetic acid (IAA) production

All the 35 isolates were screened for the IAA production in *in vitro.* Development of pink to red colour upon the addition of Salkowski reagent, indicated production of IAA (Plate 8). Based on the intensity of the colour developed, the isolates were grouped into four categories: excellent, good, moderate and poor (Table 9 and Plate 9). Details of the number of isolates under each category are given in Table 8. Three isolates were categorized as excellent producers of IAA (Ptd-A, Ptd-E, Amb-C), one as good (Ptr-A), 13 under moderate and 18 under poor. Since all the isolates were positive for IAA production, quantity of IAA was estimated under *in vitro* conditions.

#### 4.4.1.1. Estimation of indole acetic acid (IAA) production

*In vitro* production of IAA by 35 isolates was estimated quantitatively (Table 8). IAA production ranged from 0.43 μg $ml^{-1}$ to 15.9 μg $ml^{-1}$. The isolates Ptd-A and Amb-C were found to be significantly superior to all other isolates, with IAA production of 15.9 μg $ml^{-1}$ and 15.38 μg $ml^{-1}$ respectively. The lowest IAA production (0.43 μg $ml^{-1}$) was recorded by the isolate Amb-B (Table 8).

### 4.4.2. Screening of actinobacterial isolates for nitrogen fixation

All the 35 isolates were screened for nitrogen fixation on N free medium (Table 8 and plate 10). A total of seven isolates (Ptd-A, Ptd-B, Ptd-E, Amb-B, Vlt-C, Ptr-A and Ptr-D) recorded excellent growth, 16 isolates exhibited good growth and 10 isolates showed moderate growth. Two isolates (Ptr-B and Plp-B) recorded poor growth on N-free medium, indicating their poor ability to fix atmospheric nitrogen (Table 10).

#### 4.4.2.1. Estimation of nitrogen fixation

Among 35 isolates screened for growth on N-free media, seven isolates which exhibited high amount of growth were tested for the amount of nitrogen fixed, by the micro-Kjeldahl method (Table 11). Four isolates *viz.* Ptd-A, Ptd-E, Ptd-B and Ptr-A recorded significantly superior nitrogen fixation of 24.7 mg (by Ptd-A and Ptd-E), 23.3 mg (Ptd-B) and 22.87 mg (Ptr-A).

### 4.4.3. Screening of actinobacterial isolates for phosphate solubilization

All the 35 isolates were screened for phosphate solubilization (Table 8), on Pikovskaya's agar. Six isolates exhibited clear zones around the colonies (Table 12 and Plate 11). The amount of P solubilized by these six isolates (Ptd-A, Ptd-B, Ptd-D, Ptd-E, Ptr-A and Ptr-E) was further quantified.

#### 4.4.3.1. Estimation of phosphate solubilisation by actinobacterial isolates

Six phosphate solubilizing actinobacterial isolates were tested for the quantity of P solubilized and the data are presented in Plate 12. Amount of phosphate solubilized was significantly higher in Ptd-E (40.74 μg $ml^{-1}$), Ptd-D (40.46 μg $ml^{-1}$), Ptr-E (40.09 μg $ml^{-1}$), Ptd-A (39.64 μg $ml^{-1}$) and Ptr-A (38.89 μg $ml^{-1}$), as compared to the isolate Ptd-B, which solubilized 31.55 μg $ml^{-1}$ P (Table 13). All the isolates reduced the pH of the broth and lowermost pH was observed in two isolates *viz.* Ptd-D and Ptd-E.

### 4.4.4. Screening of actinobacterial isolates for potassium solubilization

None of the isolates showed the ability for potassium solubilization under *in vitro* conditions (Table 8).

### 4.4.5. Screening of actinobacterial isolates for zinc solubilization

None of the isolates showed the ability for zinc solubilization under *in vitro* conditions (Table 8).

Based on *in vitro* screening of isolates for plant growth promoting activities, three predominant isolates were selected Ptd-E, Ptd-A and Ptr-A for *in planta* evaluation.

**Table 8. Plant growth promoting activities of actinobacterial isolates under *in vitro* conditions**

| Sl. No. | Isolates | IAA production | | Growth on N free medium** (6 DAI) | Mineral solubilisation efficiency (%) | | |
|---|---|---|---|---|---|---|---|
| | | Rating | Concentration (µg $ml^{-1}$) * | | Phosphate | Potassium | Zinc |
| 1 | Ptd-A | ++++ | 15.90 (1.32)$^{a}$ | ++++ | 36.36 | - | - |
| 2 | Ptd-B | + | 2.97 (0.90)$^{ijkl}$ | ++++ | 16.67 | - | - |
| 3 | Ptd-C | + | 1.19 (0.79)$^{pqr}$ | ++ | - | - | - |
| 4 | Ptd-D | ++ | 3.27 (0.92)$^{hij}$ | +++ | 25.00 | - | - |
| 5 | Ptd-E | ++++ | 13.53 (1.27)$^{b}$ | ++++ | 50.00 | - | - |
| 6 | Amb-A | ++ | 6.35 (1.06)$^{f}$ | ++ | - | - | - |
| 7 | Amb-B | + | 0.43 (0.74)$^{st}$ | ++++ | - | - | - |
| 8 | Amb-C | ++++ | 15.38 (1.31)$^{a}$ | ++ | - | - | - |
| 9 | Amb-D | + | 2.23 (0.86)$^{mn}$ | ++ | - | - | - |
| 10 | Amb-E | + | 1.76 (0.83)$^{nop}$ | +++ | - | - | - |
| 11 | Amb-F | ++ | 8.47 (1.13)$^{d}$ | +++ | - | - | - |
| 12 | Amb-G | + | 1.49 (0.81)$^{op}$ | +++ | - | - | - |
| 13 | Amb-H | + | 2.01 (0.85)$^{mno}$ | +++ | - | - | - |
| 14 | Plt-A | ++ | 3.17 (0.91)$^{hijk}$ | +++ | - | - | - |

| Sl. No. | Isolates | IAA production | | Growth on N free medium** (6 DAI) | Mineral solubilisation efficiency (%) | | |
|---|---|---|---|---|---|---|---|
| | | Rating | Concentration (µg $ml^{-1}$) * | | Phosphorus | Potassium | Zinc |
| 15 | Vlt-B | ++ | 4.14 (0.96)$^{g}$ | +++ | - | - | - |
| 16 | Vlt-C | + | 2.52 (0.88)$^{klm}$ | ++++ | - | - | - |
| 17 | Vlt-D | + | 1.56 (0.82)$^{op}$ | ++ | - | - | - |
| 18 | Vlt-E | ++ | 3.80 (0.94)$^{h}$ | +++ | - | - | - |
| 19 | Vlt-F | + | 2.05 (0.85)$^{mno}$ | +++ | - | - | - |
| 20 | Vlt-G | + | 2.30 (0.86)$^{lmn}$ | ++ | - | - | - |
| 21 | Vlt-H | + | 2.55 (0.88)$^{jklm}$ | +++ | - | - | - |
| 22 | Vlt-I | + | 2.70 (0.89)$^{ijklm}$ | +++ | - | - | - |
| 23 | Vlt-J | + | 0.32 (0.73)$^{t}$ | +++ | - | - | - |
| 24 | Vlt-K | ++ | 7.18 (1.09)$^{def}$ | +++ | - | - | - |
| 25 | Vlt-L | ++ | 4.35 (0.97)$^{g}$ | ++ | - | - | - |
| 26 | Ptr-A | +++ | 11.22 (1.21)$^{c}$ | ++++ | 46.15 | - | - |
| 27 | Ptr-B | + | 0.86 (0.77)$^{qrs}$ | + | - | - | - |
| 28 | Ptr-C | ++ | 3.31 (0.92)$^{hi}$ | +++ | - | - | - |
| 29 | Ptr-D | ++ | 7.93 (1.11)$^{de}$ | ++++ | - | - | - |
| 30 | Ptr-E | ++ | 4.10 (0.96)$^{g}$ | ++ | 40.00 | - | - |

| Sl. No. | Isolates | IAA production | | Growth on N free medium** (6 DAI) | Mineral solubilisation efficiency (%) | | |
|---|---|---|---|---|---|---|---|
| | | Rating | Concentration (µg $ml^{-1}$) * | | Phosphorus | Potassium | Zinc |
| 31 | Ptr-F | + | 1.37 (0.80)$^{pq}$ | +++ | - | - | - |
| 32 | Plp-A | ++ | 6.96 (1.08)$^{ef}$ | ++ | - | - | - |
| 33 | Plp-B | + | 0.72 (0.76)$^{rst}$ | + | - | - | - |
| 34 | Plp-C | + | 1.17 (0.79)$^{pqr}$ | +++ | - | - | - |
| 35 | Mpd-A | ++ | 6.64 (1.07)$^{f}$ | ++ | - | - | - |

*Log transformed [log (x+5)] alues given in parantheses

**DAI- Days after incubation

++++ Excellent
+++ Good
++ Moderate
+ Poor
- No growth

**Table 9. Screening of actinobacterial isolates for indole acetic acid (IAA) production**

| SI. NO | IAA production | Isolates |
|---|---|---|
| 1 | Excellent | Ptd-A, Ptd-E, Amb-C |
| 2 | Good | Ptr-A |
| 3 | Moderate | Ptd-D, Amb-A, Amb-F, Vlt-A, Vlt-B, Vlt-E, Vlt-K, Vlt-L, Ptr-C, Ptr-D, Ptr-E, Plp-A, Mpd-A |
| 4 | Poor | Ptd-B, Ptd-C, Amb-B, Amb-D, Amb-E, Amb-G, Amb-H, Vlt-C, Vlt-D, Vlt-F, Vlt-G, Vlt-H, Vlt-I, Vlt-J, Ptr-B, Ptr-F, Plp-B, Plp-C |

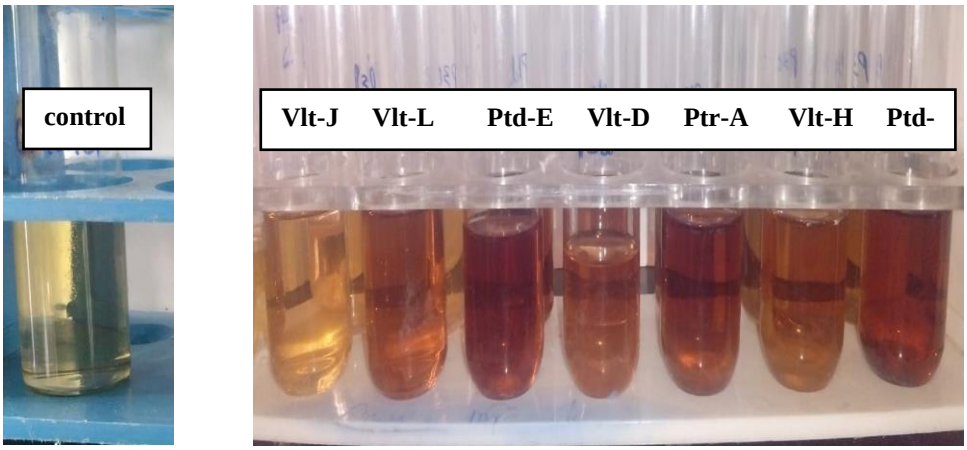


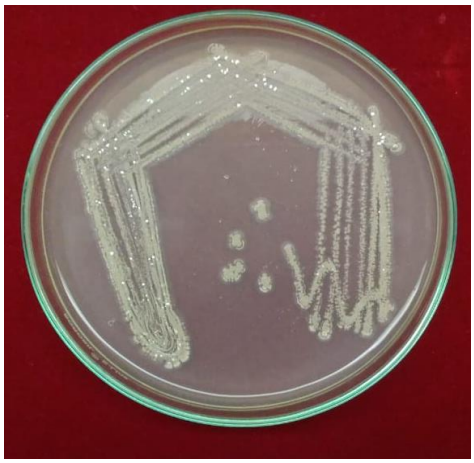

**Plate 9. Screening of actinobacterial isolates for indole acetic acid (IAA) production**

**Table 10. Screening of actinobacterial isolates for nitrogen fixation**

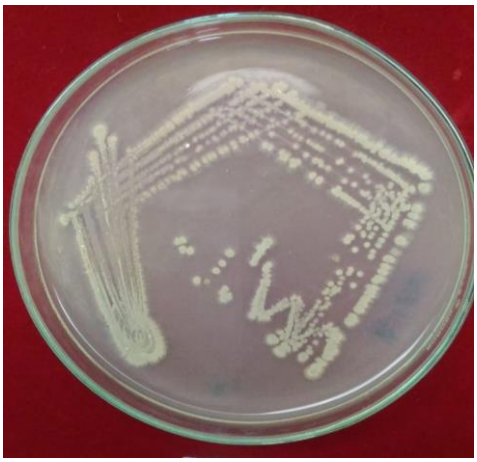
| SI. NO | Growth on N free medium | Isolates |
|---|---|---|
| 1 | Excellent | Ptd-A, Ptd-B, Ptd-E, Amb-B, Vlt-C, Ptr-A, Ptr-D |
| 2 | Good | Ptd-D, Amb-E, Amb-F, Amb-G, Amb-H, Vlt-A,Vlt-B, Vlt-E, Vlt-F, Vlt-H, Vlt-I, Vlt-J, Vlt-K, Ptr-C, Ptr-F, Plp-C |
| 3 | Moderate | Ptd-C, Amb-A, Amb-C, Amb-D, Vlt-D, Vlt-G, Vlt-L, Ptr-E, Plp-A, Mpd-A |
| 4 | Poor | Ptr-B, Plp-B |

**Table 11. Nitrogen fixation by selected actinobacterial isolates**

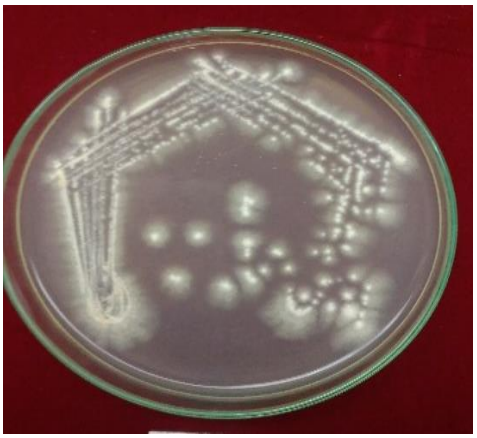
| Isolates | Amount of nitrogen fixed (mg of N $g^{-1}$ of C utilised) |
|---|---|
| Ptd-A | 24.70[a] |
| Vlt-C | 19.74[c] |
| Ptr-C | 19.97[bc] |
| Ptd-E | 24.70[a] |
| Ptd-B | 23.30[a] |
| Amb-B | 20.11[bc] |
| Ptr-A | 22.87[ab] |



**Amb-B**



**Ptr-F**



**Amb-G**

**Amb-D**

**Vlt-C**

**Amb-L**

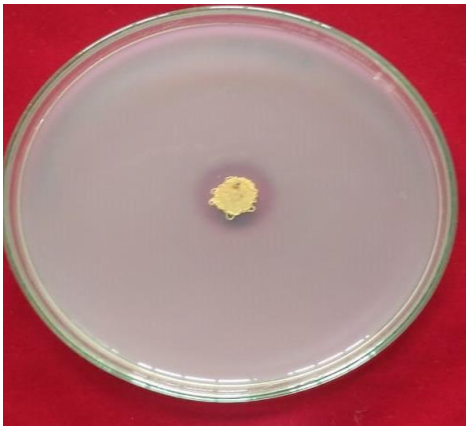
**Plate 10. Nitrogen fixing actinobacteria on N free jensen's agar**

**Table 12. Screening of actinobacterial isolates for phosphate solubilization**

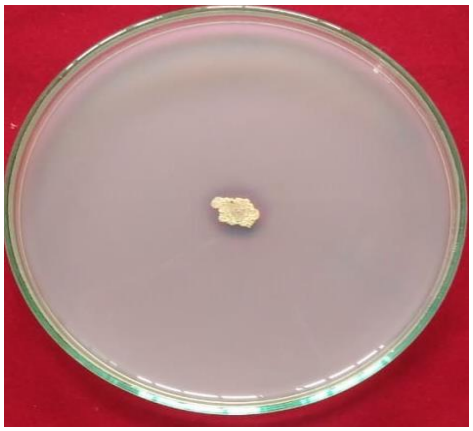
| Isolates | Phosphate solubilizing efficiency (%) |
|---|---|
| Ptd-A | 36.36 |
| Ptd-D | 25.00 |
| Ptd-E | 50.00 |
| Ptd-B | 16.67 |
| Ptr-A | 46.15 |
| Ptr-E | 40.00 |

**Table 13. Phosphate solubilization by selected actinobacterial isolates under *in vitro* conditions**

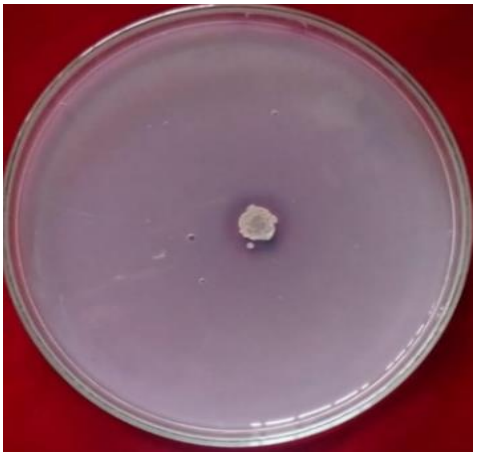
| Isolates | Quantity of P-solubilized (µg $ml^{-1}$) | Reduction in pH |
|---|---|---|
| Ptd-A | 39.64[a] | 6.0 |
| Ptd-D | 40.46[a] | 5.7 |
| Ptd-E | 40.74[a] | 5.7 |
| Ptd-B | 31.55[b] | 6.1 |
| Ptr-A | 38.89[a] | 6.0 |
| Ptr-E | 40.09[a] | 5.8 |



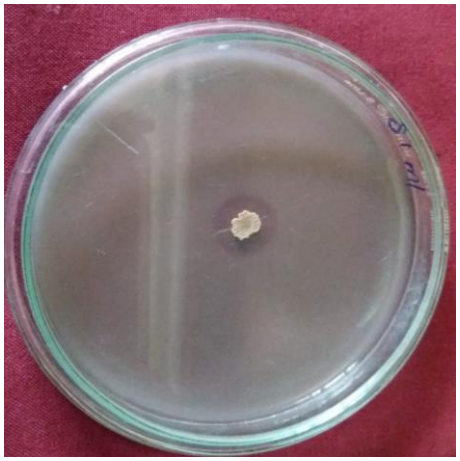
**Ptr-A**



**Ptr-E**



**Ptd-A**



**Ptd-E**

**Ptd-D**

**Ptd-B**

**Plate 11. Phosphorus solubilizing actinobacteria on pikovskaya's agar**

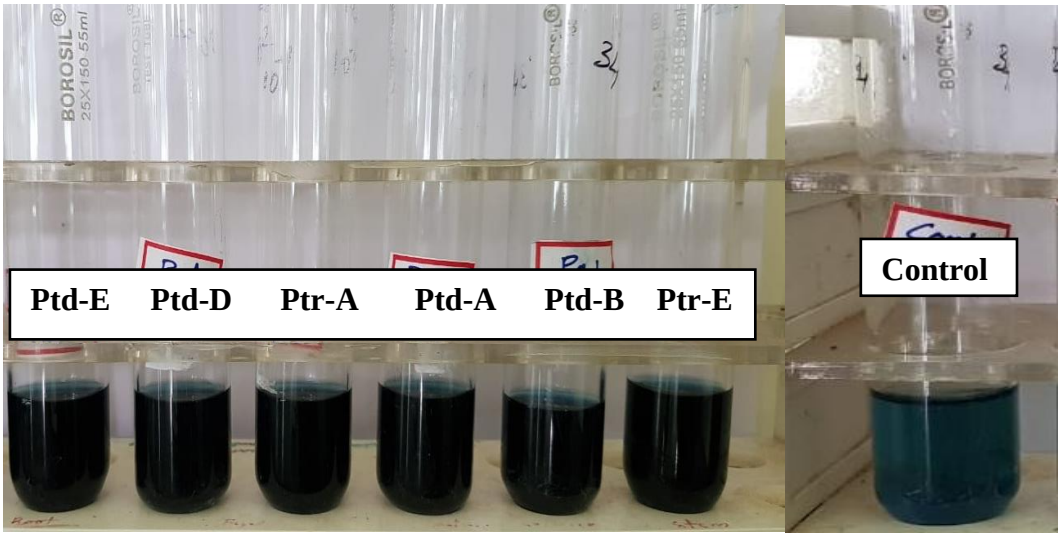



**Plate 12. Estimation of phosphate solubilisation by actinobacterial isolates**

## 4. 5. SELECTION OF EFFICIENT PLANT GROWTH PROMOTING RHIZOBACTERIA FOR FURTHER IDENTIFICATION AND EVALUATION *IN PLANTA*

Eleven isolates of actinobacteria were arranged on the basis of their IAA production, in decreasing order. A score of 10 was allotted to each of the traits and sum of scores was considered for ranking of the isolates on the basis of efficiency in plant growth promotion, from one to eleven (Table 14). The isolates with first three ranks were selected as the most efficient ones and were used for further identification by 16S rRNA sequencing followed by *in planta* evaluation.

**Table 14. Ranking of plant growth promoting isolates of actinobacteria based on efficiency under *in vitro* conditions**

| Isolates | IAA production (µg $ml^{-1}$) | Amount of nitrogen fixed (mg of N $g^{-1}$ of C utilised) | Phosphate solubilization (µg $ml^{-1}$) | Total score | Rank based on total score |
|---|---|---|---|---|---|
| **Ptd-A** | 15.90 | 24.70 | 39.64 | 802.4 | 1 |
| **Amb-C** | 15.38 | 7.11 | 0.00 | 224.9 | 7 |
| **Ptd-E** | 13.53 | 24.70 | 40.74 | 789.7 | 2 |
| **Ptr-A** | 11.22 | 22.87 | 38.89 | 729.8 | 3 |
| **Amb-F** | 8.47 | 11.08 | 0.00 | 195.5 | 11 |
| **Ptr-E** | 4.10 | 8.23 | 40.09 | 524.2 | 5 |
| **Ptr-C** | 3.39 | 19.97 | 0.00 | 233.6 | 8 |
| **Ptd-D** | 3.27 | 14.55 | 40.46 | 582.8 | 4 |
| **Ptd-B** | 2.97 | 13.67 | 31.55 | 481.9 | 6 |
| **Vlt-C** | 2.52 | 19.74 | 0.00 | 222.6 | 9 |
| **Amb-B** | 0.43 | 20.11 | 0.00 | 205.4 | 10 |

## 4.6. IDENTIFICATION OF SELECTED ACTINOBACTERIA BY MOLECULAR CHARACTERIZATION

Molecular characterization of selected actinobacteria was carried out by 16S rRNA gene sequencing. PCR amplified products on one per cent agarose gel appeared as crisp bands of 1500 bp size. Nucleotide sequences obtained from Rajiv Gandhi Centre for Biotechnology, Trivandrum were used for homology search using BLASTn (Plates 13, 14 and 15). The sequence analysis of the isolate Ptd-A showed 98 per cent query coverage and 99.45 per cent identity to *Streptomces* sp. (NCBI accession No. JF806660.1). The isolate Ptd-E showed 98 per cent query coverage and 98.75 per cent identity with *Actinobacteria bacterium* (NCBI accession No. MK424352.1). Sequence analysis of the isolate Ptr-A showed 99 per cent query coverage and 98.98 per cent identity to *Streptomyces* sp. (NCBI accession No. MF344821.1). Based on 16S rRNA gene sequencing, the isolates were identified and the sequences were deposited in the GenBank of the NCBI. Accession numbers were received from GenBank for respective isolates (Table 15), as Ptd-A: *Streptomyces* sp. (NCBI accession No. MW534685), Ptd-E: *Actinobacteria bacterium* (NCBI accession No. MW534690) and Ptr-A: *Streptomyces* sp. (NCBI accession No. MW534694). This was also in conformity with cultural, morphological and biochemical characterization.

## 4.7. PHYLOGENETIC TREE

Evolutionary analysis of Ptd-A, Ptd-E and Ptr-A was conducted by constructing the phylogenetic trees (Plates 13, 14 and 15). Phylogenetic analysis revealed two clusters. In first cluster, the actinobacterial isolate *Streptomyces* sp. strain Ptd-A and *Actinobacteria bacterium* strain Ptd-E were grouped together with 91 per cent bootstrap confidence value. In the second cluster, *Streptomyces* sp. strain Ptr-A was closely positioned to *Streptomyces* sp. strain MJM16245, *Streptomyces* sp. strain WI04-3A and *Streptomyces* sp. strain HF-6, with a bootstrap confidence value of 100 per cent.

TAACGTTCGGTGGGGATTAGTGGCGACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAA
GCCCTGGAAACGGGGTCTAATACCGGATATCACTCCCGCAGGCATCTGCGGGTGTCGAAAGCTCCGGCGG
TGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCC
GGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTA
AACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCT
TGTCACGTCGGGGTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCTAGCTAGAGTGT
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AGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGGAGCGAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGGTGGGAAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGCTA
ACGCATTAAGTTTCCCCGCCTGGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAGTGAACGGGGGCC
CGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACAC
CGGAAAACCCTGGAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCATGCCCTTCGGGG
TGATGGGGACTCACAGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATG
CCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGAAACCGTGAGGTGGAG
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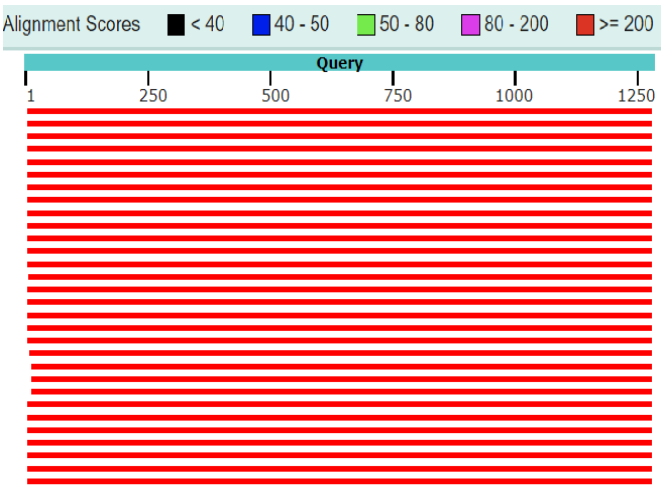
TAATCGCAGATCAGGCAAGGTG

**A. Sequence of 16S rRNA amplicon**




**B. Blastn output**

| Description | Max. score | Query coverage (%) | Accession | Identity (%) | E value |
|---|---|---|---|---|---|
| *Streptomyces* sp. | 2302 | 98 | JF806660.1 | 99.45 | 0.0 |
| *Actinobacteria bacterium* | 2294 | 98 | MK424352.1 | 99.37 | 0.0 |
| *Streptomyces hygroscopicus* | 2294 | 98 | KX358637.1 | 99.37 | 0.0 |
| *Streptomyces* sp. | 2294 | 98 | KP126297.1 | 99.37 | 0.0 |

**C. Sequence showing homology**

**Plate 13. Sequence analysis of isolate Ptd-A**

CGGTTCGGTGGGGATAGTGGCGACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCC
CTGGAAACGGGGTCTAATACCGGATATCACTCCCGCAGGCATCTGCGGGTGTCGAAAGCTCCGGCGGTGA
AGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGC
CTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAAC
CTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCA
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AGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCT
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ACGCATTAAGTTTCCCCGCCTGGGGAGTACGGCCCGCAACGCCTAAAACTTCAAAGGTAATTGACGGGGG
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ACCGGAAAACCCTGGAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCATGCCCTTCG
GGGTGATGGGGACTCACAGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATC
ATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGAAACCGTGAGGTG
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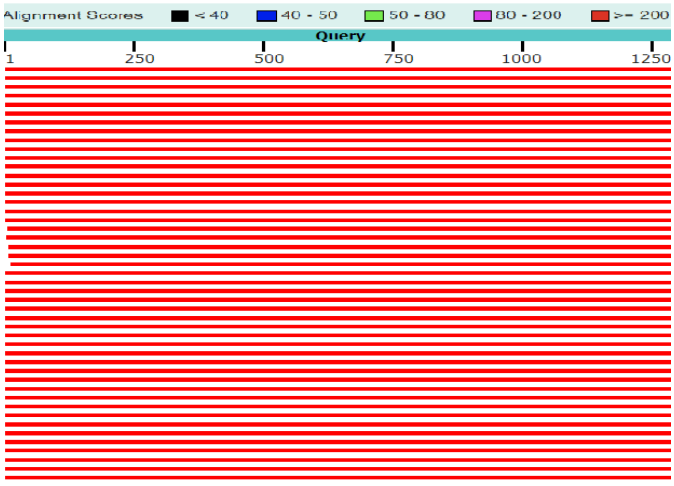
**A. Sequence of 16S rRNA amplicon**




**B. Blastn output**

| Description | Max. score | Query coverage (%) | Accession | Identity (%) | E value |
|---|---|---|---|---|---|
| *Actinobacteria bacterium* | 2259 | 98 | MK424352.1 | 98.75 | 0.0 |
| *Streptomyces hygroscopicus* | 2259 | 98 | KX358637.1 | 98.75 | 0.0 |
| *Streptomyces* sp. | 2259 | 98 | KP126297.1 | 98.75 | 0.0 |
| *Streptomyces* sp. | 2259 | 98 | KP126257.1 | 98.75 | 0.0 |

**C. Sequence showing homology**

**Plate 14. Sequence analysis of isolate Ptd-E**

ACGTTCGGTGGGGATAGTGGCGACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAA
ACGGGGTCTAATACCGGATATGACCATCTTGGGCATCCTTGATGGTGTAAAGCTCCGGCGGTGAAGGATGAGCCCG
CGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCC
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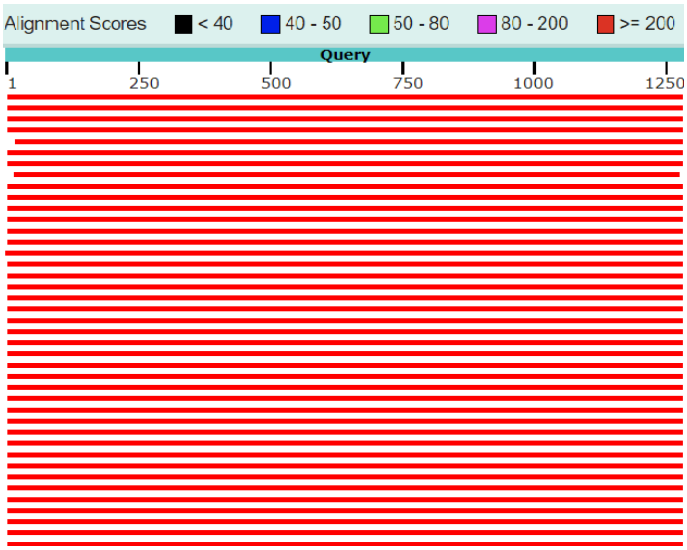
ACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCG
GATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAAGCATGGTG

**A. Sequence of 16S rRNA amplicon**




**B. Blastn output**

| Description | Max. score | Query coverage (%) | Accession | Identity (%) | E value |
|---|---|---|---|---|---|
| *Streptomyces* sp. | 2274 | 99 | MF344821.1 | 98.98 | 0.0 |
| *Streptomyces* sp. | 2274 | 99 | MH698908.1 | 98.98 | 0.0 |
| *Streptomyces* sp. | 2268 | 99 | MG846092.1 | 98.90 | 0.0 |
| *Streptomyces* sp. | 2268 | 99 | MF784500.1 | 98.90 | 0.0 |

**C. Sequence showing homology**

**Plate 15. Sequence analysis of isolate Ptr-A**

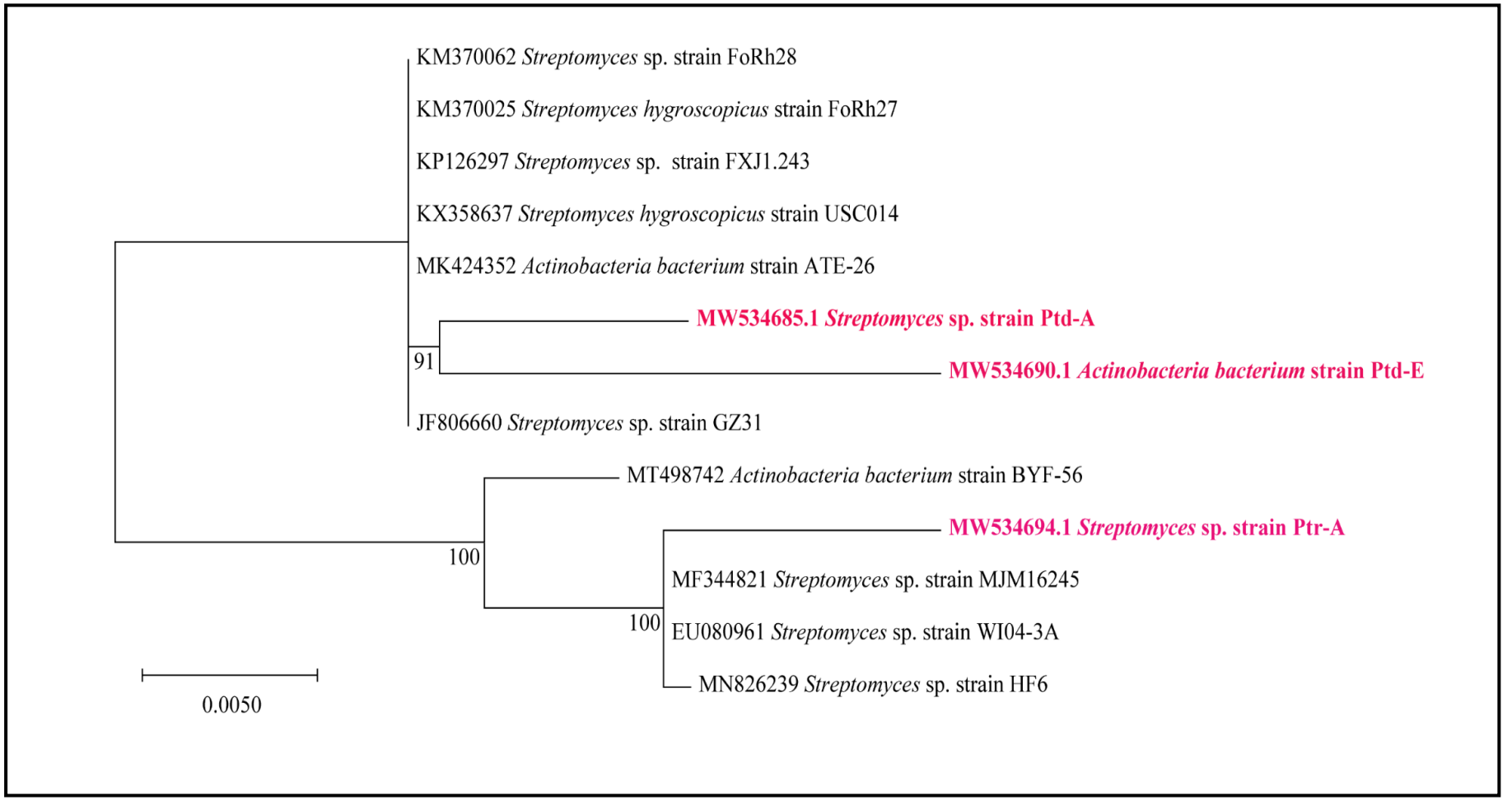



**Fig 2. Phylogenetic tree of selected actinobacterial isolates with other members of genera *Streptomyces* and *Actinobacteria* on the basis of 16S rRNA gene sequencing**

**Table 15. Plant growth promoting characters of selected actinobacterial isolates**

| SI. No | Isolates | Location | Accession | IAA (µg $ml^{-1}$) | N fixation (mg of N $g^{-1}$ of C utilised) | Phosphate solubilization (µg $ml^{-1}$) |
|---|---|---|---|---|---|---|
| 1 | Ptd-A: *Streptomyces* sp. | Puttad | MW534685 | 15.9 | 24.70 | 39.64 |
| 2 | Ptd-E: *Actinobacteria bacterium* | Puttad | MW534690 | 13.53 | 24.70 | 40.74 |
| 3 | Ptr-A: *Streptomyces* sp. | Pathiri | MW534694 | 11.22 | 22.87 | 38.89 |

## 4.8. EVALUATION OF SELECTED ACTINOBACTERIA FOR GROWTH PROMOTION IN BLACK PEPPER CUTTINGS

Three efficient actinobacteria having multiple PGP traits were evaluated for plant growth promotion in one month old black pepper rooted cuttings.

Treatments:

$T_1$: Ptd-E (*Actinobacteria bacterium*)

$T_2$: Ptd-A (*Streptomyces* sp.)

$T_3$: Ptr-A (*Streptomyces* sp.)

$T_4$: PGPR Mix-1

$T_5$: Organic POP recommendation of KAU (2017)

(Organic POP recommendation included 1.5 kg FYM + 25 g neem cake+ 1 handful of ash+ 2 handful of vermicompost+ 2g *Azospirillum* + 2g phosphorus solubilizing bacteria + 5 g PGPR consortia per polybag)

$T_6$: Absolute control

### 4.8.1. Population of actinobacterial isolates in sterile potting mixture

The population of actinobacteria in potting mixture planted with black pepper was assessed by serial dilution and plating technique (Table 16) at various intervals *ie.* 30 DAP, 75 DAP, 105 DAP and 150 DAP.

In the potting mixture, the presence of actinobacteria was detected in the treatments $T_1$, $T_2$ and $T_3$ at all the intervals tested. The population of actinobacteria ranged from 3.6 x$10^6$ cfu $g^{-1}$ to 6.67 x$10^6$ cfu $g^{-1}$ and were statistically on par in all these treatments. No actinobacteria could be detected in the treatments $T_4$, $T_5$ and $T_6$ at 30, 75, 105 and 150 days of planting.

### 4.8.2. Biometric characters of black pepper

#### 4.8.2.1. Vine length

Data on vine length of black pepper are presented in Table 18. At one month after planting (MAP), significantly higher vine length was recorded in treatments $T_2$ (20.50 cm), $T_1$ (19.29 cm) and $T_3$ (18.74 cm) than the other treatments. Vine length was significantly lower in $T_6$ (9.17 cm), which was on par with $T_5$ (10.60 cm).

Similar trend was observed at the other intervals also. $T_1$, $T_2$ and $T_3$ recorded significantly higher vine length at all the intervals. The lowest vine length was recorded for $T_6$ (Absolute control) at two and three MAP, with 18.15 cm and 23.18 cm respectively. These values were statistically on par with vine length in $T_5$ (32.37 cm and 35.25 cm at two and three MAP respectively) (Plate 17).

At four and five months after planting, significantly lower vine length was recorded in $T_6$ (32.37 cm and 35.25 cm respectively).

#### 4.8.2.2. Number of leaves

The data on number of leaves per black pepper plant at various intervals are given in Table 18. After 30 days of planting, $T_2$ (5.46), $T_3$ (5.457) and $T_1$ (4.96) recorded significantly higher number of leaves per plant. Significantly lower number of leaves was observed in $T_6$ (2.48) and $T_5$ (2.79).

Similar trend was observed at two, three, four and five MAP. Treatments $T_1$, $T_2$ and $T_3$ were significantly superior to the other treatments. Minimum number of leaves

**At the time of planting**


**5 MAP**

**Plate 16. An overview of pot culture experiment**

observed for $T_6$ and $T_5$ (Plate 17) at two and three MAP. At five MAP, significantly lower number of leaves per plant was observed in $T_6$.

#### 4.8.2.3. Internode length

The data on internode length of pepper vines are presented in Table 19. No significant differences were noticed up to two months after planting. At three MAP, $T_2$ (5.55 cm), $T_3$ (5.45 cm), $T_1$ (5.25 cm) and $T_4$ (5.33 cm) were significantly superior to other treatments. Significantly lower internode length was observed for $T_6$ with 4.10 cm and $T_5$ with 4.51 cm. Similar trend was observed at four and five months after planting also (Plate 17).

#### 4.8.2.4. Root volume

After five months after planting, maximum root volume was observed in $T_2$ with 3.54 ml. This was significantly superior to all other treatments. Minimum root volume was observed in $T_6$ and $T_5$ with values of 1.25 ml and 1.75 ml respectively (Table 20).

#### 4.8.2.5. Fresh weight of roots

Five months after planting, significantly higher fresh weight of roots was observed in $T_2$ (4.31 g). This was statistically superior to all other treatments. Minimum root weight was observed in $T_6$ with 1.52 g (Table 20 and plate 18).

#### 4.8.2.6. Dry weight of roots

At five MAP, maximum dry weight of roots was observed in $T_2$ with 1.23g, and this was statistically superior to all other treatments. This was followed by $T_3$ and $T_1$ with 0.92 g and 0.80g respectively. Minimum dry root weight was observed in $T_6$ with 0.51 g and $T_5$ (0.64 g) (Table 20 and Plate 18).

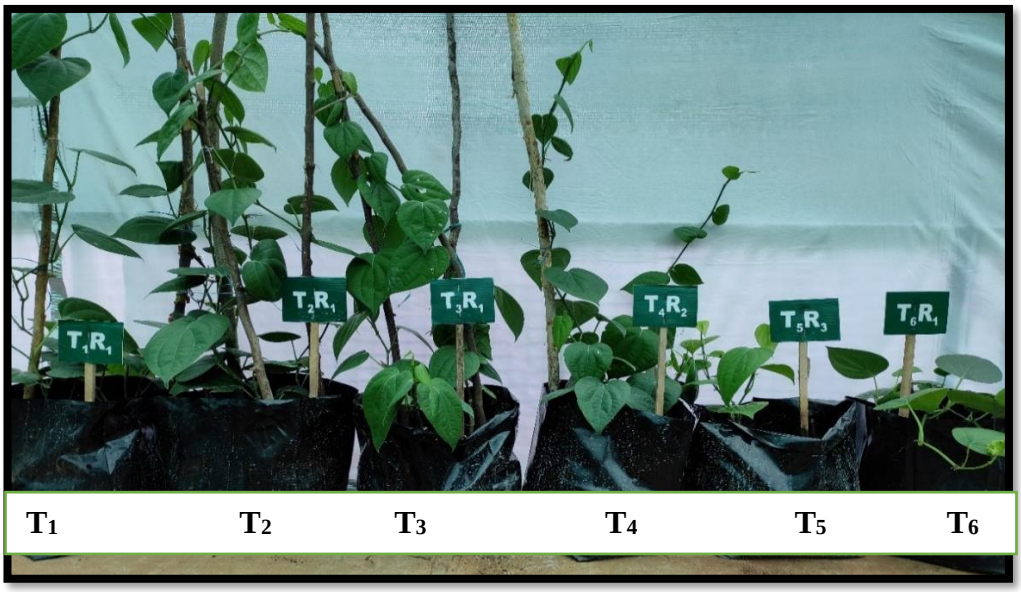
#### 4.8.2.7. Fresh weight of plants

Five months after planting, significantly higher fresh weight of plant was observed in $T_2$ (52.52 g) and this was statistically superior to all other treatments. This was followed by $T_3$ and $T_1$ with 48.02 g and 44.57 g respectively. Minimum plant weight was observed in $T_6$ with 8.39 g (Table 21 and Plate 19).

#### 4.8.2.8. Dry weight of plants

At five MAP, maximum dry weight of plants was observed in $T_2$ (13.50g) and $T_3$ (12.24 g), which were statistically on par. Minimum dry plant weight was observed in $T_6$ with 3.35 g (Table 21 and Plate 19).


**1 MAP**




**5 MAP**

**Plate 17. Effect of PGPM on plant growth**

**Table 16. Population of actinobacteria in potting mixture at different intervals**

| Sl. No | Treatments | Population of total actinobacteria (x$10^6$ cfu $g^{-1}$) | | | |
|---|---|---|---|---|---|
| | | 30 DAP | 75 DAP | 105 DAP | 150 DAP |
| 1 | $T_1$: Ptd-E *Actinobacteria bacterium* | 6.67 (0.87)[a] | 5.00 (0.73)[a] | 3.67 (0.65)[a] | 4.00 (0.67)[a] |
| 2 | $T_2$: Ptd-A *Streptomyces* sp. | 4.00 (0.64)[a] | 5.33 (0.76)[a] | 4.67 (0.75)[a] | 4.00 (0.68)[a] |
| 3 | $T_3$: Ptr-A *Streptomyces* sp. | 6.00 (0.83)[a] | 6.00 (0.83)[a] | 4.33 (0.70)[a] | 4.33 (0.72)[a] |
| 4 | $T_4$: PGPR Mix 1 | 0.00[b] | 0.00[b] | 0.00[b] | 0.00[b] |
| 5 | $T_5$: Organic POP, 2017 | 0.00[b] | 0.00[b] | 0.00[b] | 0.00[b] |
| 6 | $T_6$: Control | 0.00[b] | 0.00[b] | 0.00[b] | 0.00[b] |

Log transformed values in parantheses

**Table 17. Effect of actinobacteria on vine length of black pepper**

| Sl. No | Treatments | Black pepper vine length (cm) | | | | |
|---|---|---|---|---|---|---|
| | | 1 MAP (cm) | 2 MAP (cm) | 3 MAP (cm) | 4 MAP (cm) | 5 MAP (cm) |
| 1 | $T_1$: Ptd-E (*Actinobacteria bacterium*) | 18.74[a] | 25.58[ab] | 46.99[ab] | 61.55[a] | 70.82[a] |
| 2 | $T_2$: Ptd-A (*Streptomyces* sp.) | 20.50[a] | 29.14[a] | 56.46[a] | 69.22[a] | 77.49[a] |
| 3 | $T_3$: Ptr-A (*Streptomyces* sp.) | 19.29[a] | 26.98[a] | 55.85[a] | 68.58[a] | 74.75[a] |
| 4 | $T_4$: PGPR Mix 1 | 12.26[b] | 23.76[bc] | 39.83[bc] | 44.67[b] | 52.53[b] |
| 5 | $T_5$: Organic POP, 2017 | 10.60[bc] | 20.08[cd] | 33.91[cd] | 42.38[b] | 47.61[b] |
| 6 | $T_6$: Control | 9.17[c] | 18.15[d] | 23.18[d] | 32.37[c] | 35.25[c] |


T$_1$: *Actinobacteria bacterium* Ptd-E


T$_2$: *Streptomyces* sp. Ptd-A


T$_3$: *Streptomyces* sp. Ptr-A


T$_4$: PGPR Mix- 1


T$_5$: Organic POP recommendation (2017)

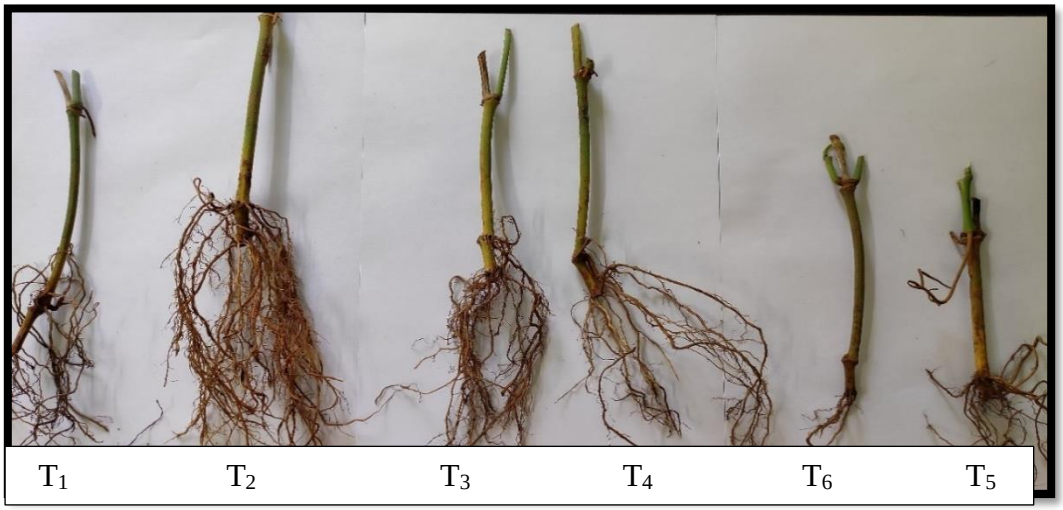
**Plate 18. Effect of actinobacteria on plant growth**

**Table 18. Effect of actinobacteria on number of leaves of black pepper**

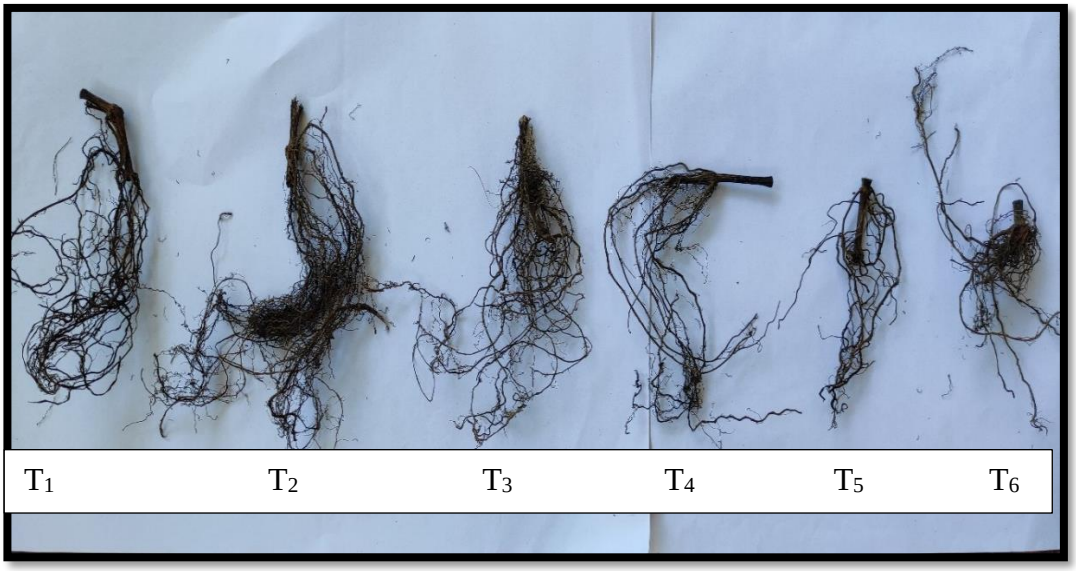
| Sl. No | Treatments | No. of leaves per plant | | | | |
|---|---|---|---|---|---|---|
| | | 1 MAP | 2 MAP | 3 MAP | 4 MAP | 5 MAP |
| 1 | $T_1$: Ptd-E (*Actinobacteria bacterium*) | 4.97[a] | 6.46[a] | 9.07[ab] | 12.09[a] | 13.42[a] |
| 2 | $T_2$: Ptd-A (*Streptomyces* sp.) | 5.46[a] | 6.99[a] | 10.26[a] | 13.69[a] | 15.01[a] |
| 3 | $T_3$: Ptr-A (*Streptomyces* sp.) | 5.46[a] | 6.79[a] | 10.07[a] | 12.67[a] | 14.04[a] |
| 4 | $T_4$: PGPR Mix 1 | 3.14[b] | 5.034[b] | 7.60[bc] | 8.43[b] | 9.62[b] |
| 5 | $T_5$: Organic POP, 2017 | 2.79[b] | 4.38[bc] | 5.59[cd] | 7.55[b] | 8.91[b] |
| 6 | $T_6$: Control | 2.48[b] | 3.55[c] | 4.13[d] | 4.60[c] | 5.47[c] |

**Table 19. Effect of actinobacteria on internode length of black pepper**

| Sl. No | Treatments | Internode length of black pepper vines (cm) | | | | |
|---|---|---|---|---|---|---|
| | | 1 MAP | 2 MAP | 3 MAP | 4 MAP | 5 MAP |
| 1 | $T_1$: Ptd-E (*Actinobacteria bacterium*) | 3.97 | 4.29 | $5.25^{ab}$ | $5.70^{ab}$ | $5.85^{ab}$ |
| 2 | $T_2$: Ptd-A (*Streptomyces* sp.) | 3.89 | 4.19 | $5.55^{a}$ | $6.32^{a}$ | $6.47^{a}$ |
| 3 | $T_3$: Ptr-A (*Streptomyces* sp.) | 3.74 | 3.96 | $5.45^{a}$ | $6.12^{a}$ | $6.32^{a}$ |
| 4 | $T_4$: PGPR Mix 1 | 3.62 | 3.71 | $5.33^{a}$ | $5.65^{ab}$ | $5.83^{ab}$ |
| 5 | $T_5$: Organic POP, 2017 | 3.52 | 3.65 | $4.51^{bc}$ | $5.06^{bc}$ | $5.16^{bc}$ |
| 6 | $T_6$: Control | 3.24 | 3.51 | $4.10^{c}$ | $4.66^{c}$ | $4.73^{c}$ |




**Root architecture in different treatments**




**Dried roots in different treatments**

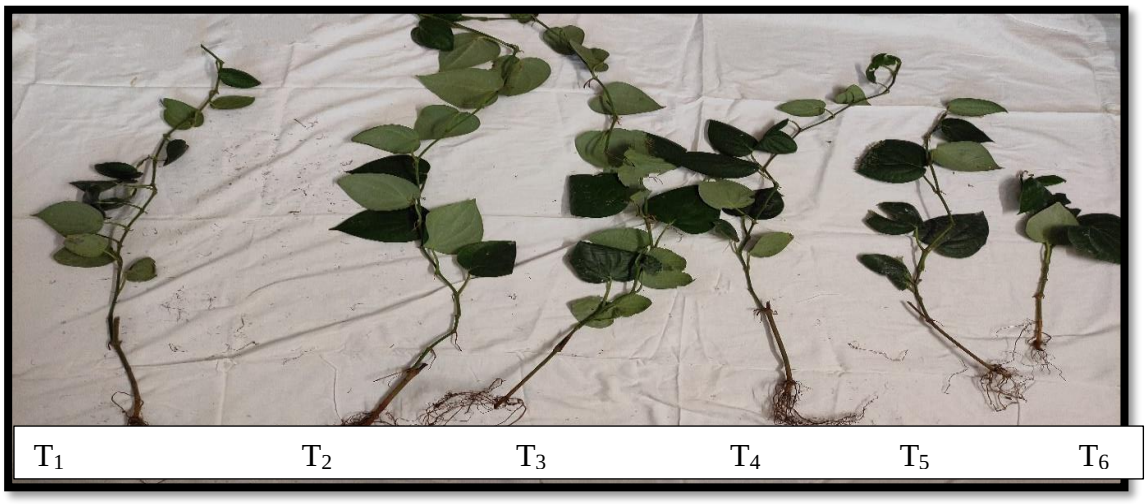
**Plate 19. Effect of actinobacteria on root growth**

**Table 20. Effect of actinobacteria on root growth of black pepper**

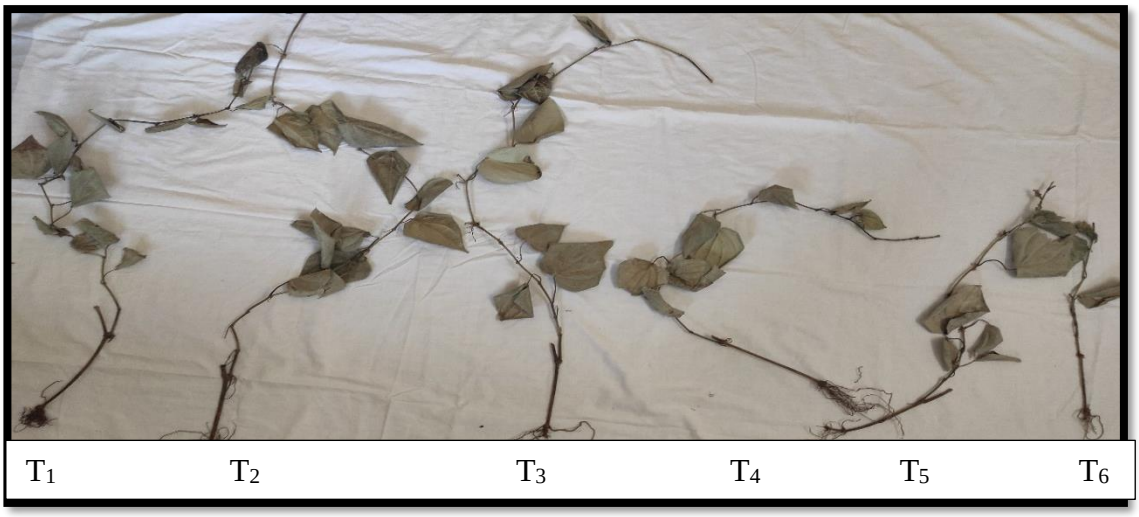
| Sl. No | Treatments | Root volume (ml/plant) | Fresh weight (g/plant) | Dry weight (g/plant) |
|---|---|---|---|---|
| 1 | $T_1$: Ptd-E (*Actinobacteria bacterium*) | $2.59^{b}$ | $3.29^{bc}$ | $0.80^{bc}$ |
| 2 | $T_2$: Ptd-A (*Streptomyces* sp.) | $3.54^{a}$ | $4.31^{a}$ | $1.23^{a}$ |
| 3 | $T_3$: Ptr-A (*Streptomyces* sp.) | $2.69^{b}$ | $3.65^{b}$ | $0.92^{b}$ |
| 4 | $T_4$: PGPR Mix 1 | $1.99^{bc}$ | $2.81^{cd}$ | $0.70^{c}$ |
| 5 | $T_5$: Organic POP, 2017 | $1.75^{cd}$ | $2.21^{d}$ | $0.64^{cd}$ |
| 6 | $T_6$: Control | $1.25^{d}$ | $1.52^{e}$ | $0.51^{d}$ |

**Table 21. Effect of actinobacteria on fresh and dry weight of black pepper plants**

| Sl. No | Treatments | Fresh weight (g/plant) | Dry weight (g/plant) |
|---|---|---|---|
| 1 | $T_1$: Ptd-E (*Actinobacteria bacterium*) | $44.57^{b}$ | $11.25^{b}$ |
| 2 | $T_2$: Ptd-A (*Streptomyces* sp.) | $52.52^{a}$ | $13.50^{a}$ |
| 3 | $T_3$: Ptr-A (*Streptomyces* sp.) | $48.02^{b}$ | $12.24^{ab}$ |
| 4 | $T_4$: PGPR Mix 1 | $29.53^{c}$ | $9.61^{c}$ |
| 5 | $T_5$: Organic POP, 2017 | $19.64^{d}$ | $7.19^{d}$ |
| 6 | $T_6$: Control | $8.39^{e}$ | $3.35^{e}$ |




**Fresh plants in different treatments**




**Dried plants in different treatments**

**Plate 20. Effect of actinobacteria on fresh and dry weight of black pepper plants**

# *5. Discussion*

# 5. DISCUSSION

Plant growth promoting rhizobacteria are the soil bacteria that live in and around the root surface and are indirectly or directly involved in enhancing plant growth and development through production and emission of several regulatory chemicals in the rhizosphere area (Kloepper and Schroth, 1981). Plant rhizosphere is adaptable and active environmental atmosphere of large microbe-plant interactions for utilizing essential micro and macronutrients from the little nutrient pool. Generally, plant growth promoting rhizo-microbiome produces beneficial outcome on plant growth directly by hastening nutrient acquisition, fixation of atmospheric $N_2$, solubilization of phosphorus, potassium, zinc, production of siderophores, synthesis of plant growth hormones such as indole acetic acid, gibberellic acid, cytokinins *etc*. Indirect mechanism involves biocontrol of plant pathogens and harmful microbes, thereby they act as biocontrol agents (El-Hadad et al., 2010). In addition, these microbes and the compounds they secrete help in the remediation activity and play chief roles in altering plant stress responses.

Soil microbes probably constitute the world's prominent biological pool (Torsvik et al., 1990). The diversity and spread of soil and rhizoshere microflora depend on several elements such as root morphology, plant growth stage, root exudates, and physical and chemical properties of the soil. Many plants show genetic tolerance to rhizosphere microbes and the plant variety manages the rhizosphere microbial community. Plants can also compete with rhizosphere microorganisms for resources like water and nutrients (Sylvia, 2005). The soil microbial diversity is also linked to complication of the microbial interaction like microbe-plant and microbe-soil interaction. The plant-microbe interaction in rhizosphere has an important role in sustaining soil fertility and plant health.

In the diverse rhizosphere microorganisms, actinobacteria comprise a large part. They are one of the major parts of rhizosphere microbial inhabitants and are useful in soil nutrient cycling and plant growth promotion (PGP). As PGPR, they have both direct as well as indirect mechanisms that promote plant growth. Hence, actinobacteria can be considered as plant biofertilizers (Franco-correa and Chavarro-Anzola, 2016).

Black pepper (*Piper nigrum* L), a highly export oriented spice crop, is a perennial vine which originated in the Western Ghats of India. Black pepper cultivation is mostly focused in the southern states *i.e.,* Kerala and Karnataka. In Kerala, Wayanad is the major district growing black pepper. Black pepper export is limited due to less yield, low content of oleoresin and many diseases. Therefore, there is a felt-need to find inputs and technologies that can ensure enhanced crop productivity and support ecological balance in agro-ecosystem. Use of microbial strains or plant growth promoting rhizobacteria (PGPR) for the improvement of productivity in sustainable agriculture is an extensively accepted method in numerous parts of the world. Biofertilizer is an "ecofriendly" organic agro-input which has the potential to transform nutritionally important elements from non-available to available form through biological activities (Vessey, 2003). Combining bio-fertilizer technology with the traditional fertilizer can help to grow crops in a sustainable method.

Beneficial actinobacteria present in the rhizosphere of plants could be exploited for plant growth promotion. Not many systematic studies have been undertaken on the actinobacteria which enhance plant growth in black pepper from Wayanad. A study by Bhai et al. (2016) revealed that maximum actinobacterial isolates were obtained from Wayanad region, the most suitable area for black pepper cultivation in Kerala. Anusree and Bhai (2017), assessed the variability in the genera of actinobacteria in different rhizosphere soil samples of black pepper and observed that actinobacterial diversity was more in Wayanad, compared to other districts of Kerala.

Kerala was severely affected by flood during August-September 2018 and there is no documentation on how the flood has affected soil microorganisms including actinobacteria. Hence, the present study was an attempt to identify isolates of potential plant growth promoting actinobacteria from flood affected and non-flood affected rhizosphere soils of black pepper in Wayanad.

Ten soil samples consisting of five from non-flood affected and five from flood affected black pepper gardens in Wayanad district were assessed for actinobacterial population. Among different soil samples analysed, Puttad (Ptd) recorded significantly higher actinobacterial population on all the three media. The population of Ambalavayal soil was significantly higher on Starch Casein Agar. Lowest population was observed in Pathiri (Ptr) sample on Kenknight & Munaier's agar and actinomycetes isolation agar.

Actinobacterial colonies could not be detected in any of the flood affected soil samples on any medium, even at a dilution of $10^{-1}$, except in Meppadi soil, which recorded a very low population of $0.3x10^{1}$cfu $g^{-1}$ soil. One of the reasons for the absence of actinobacteria in flood affected soils could be the limited entry of atmospheric oxygen during flooding, which could have brought about physical, chemical and biological consequences for soil habitats and species (Graff and Conrad, 2005). Bassio and Scow (1995) reported that flooding caused a decrease in the metabolically active microbial diversity, using BIOLOG microplates. Flooding led to a decrease in the obtainability of suitable electron acceptors resulting in lower rates of organic matter decomposition (Bassio and Scow, 1995). Similar study conducted by Shiburaj (2018) revealed that flooding had affected the natural microbiota of the soil. In flooded condition, there is a depletion in soil enzyme activity compared to buffer and non-flooded areas indicating that there is a decrease in beneficial microbial diversity in flooded soil. An experiment conducted by Furtak et al. (2019) reported that the number of actinobacteria and eubacteria of the genera *Streptomyces, Arthrobacter, Arenimonas, Gaiella, Agromyces, Nocardioides, Solirubrobacter, Bacillus, Mycobacterium* decreased in flooded conditions.

Enumeration of actinobacteria was carried out on three different media *viz.* Kenknight-Munaier's agar, Actinomycete Isolation Agar and Starch Casein Agar. Any single medium may not be optimum for all the actinobacteria present in the soil, and hence three different media were used. It was also expected to assess the morphology of actinobacterial colonies on different media. It has been reported that actinobacteria are a group of slow growing microorganisms and therefore require complex media. For any commercial production of cultures, survival and uninterrupted growth are essential. This depends upon an adequate supply of nutrients and a favourable growth

environment. The usual recommended media for cultivation of actinobacteria are International Streptomyces Project (ISP) media, Kenknight media (KK) and Starch Casein Agar (SCA) media. A Modified Nutrient Agar medium with yeast extract and NaCl was developed by Ganeshamurthy et al. (2021), which was better than all other media and yielded good growth of most of the actinomycetes. In the present investigation, Starch Casein recorded higher population in all the non-flooded locations, except Pulpally. In a similar study conducted by Njenga et al. (2017) to isolate actinobacteria from soil using three isolation media (Starch Casein, Luria Bertani and Starch Nitrate Agar), the population of actinobacteria obtained was higher in Starch Casein Agar than Luria Bertani and Starch Nitrate Agar medium. In the present investigation, the morphology of colonies differed, as indicated by pigment production by the isolate Vlt-K on Kenknight and Munaier's Agar, whereas the same isolate did not produce any pigment on Starch Casein Agar medium. Thampi and Bhai, (2017) also reported difference in colony morphology of actinobacteria on different media.

Cultural, morphological, biochemical and physiological characters were used for characterization and identification of thirty-five isolates of actinobacteria, following Bergey's Manual of Determinative Bacteriology (Breed et al., 1948). The isolates varied in their colony morphology, colour of aerial mycelium and pigment production. The colour of aerial mycelium varied from dark brown to light brown, white, purple or green. Colonies were circular or irregular, with the exception of Amb-A and Plt-A, which were filamentous, and Plp-A and Plp-C which were rhizoid. The elevation, form and margin of colonies varied among the isolates. Morphological characterization of the actinobacterial isolates by different parameters such as spore chain morphology, spore colour, spore shape and spore surface ornamentation are crucial for classification of the actinobacteria (Kumar et al., 2011).

According to Burkholder et al 1954, the microscopic characterization based on aerial and substrate mycelium and spore chain morphology is a very useful tool in the identification of actinobacteria. Spore chain morphology was found to be the most constant character and clearly mentioned feature used for actinobacterial classification. In the present investigation, the spore chain morphology of 35 isolates were studied and

it was found to vary among the genera. The spore chain morphology varied among the genera and 14 isolates produced rectiflexibile type of sporulating aerial hypae *ie*. straight or flexuous spore chains, partly in fascicles. Six isolates each formed spira type, short chain and closed spira of sporulating aerial hyphae. Single spore, sporangia formation and filamentous type of aerial hyphae were observed in one isolate each. Anusree and Bhai (2017) also categorized actinobacterial spore chains as rectiflexibiles, spira, retinaculiaperti, fragments, short chain, sporangia *etc*. as observed under the light microscope.

Two isolates Vlt-K and Plp-B produced yellow water-soluble pigment and Vlt-I produced water-insoluble purple pigment on Kenknight and Munaier's Agar. Li et al. (2016) reported that actinobacteria produce two type of pigments water-soluble pigment or diffusible pigment and fat-soluble pigment or non-diffusible pigment. The isolate Vlt-K produced yellow water-soluble pigment on Kenknight and Munaier's Agar but not on Starch Casein Agar. Similarly the isolate Amb-B produced white colonies on Starch Casein Agar but on Kenknight and Munaier's Agar produced brown colonies. It has already been reported that the colony morphology depends on the media used, so it cannot be taken as basic criteria for identification of microbes (Thampi and Bhai, 2017).

Gram staining revealed that all the actinobacterial isolates obtained from Wayanad were Gram positive in nature.

The ability of 35 actinobacterial isolates to ferment sugars and produce acids from various carbohydrate sources such as glucose, sucrose and fructose was tested. Three isolates (Ptd-A, Vlt-B and Vlt-G) produced a colour change of the broth from red to yellow, without any gas formation, which indicated that they are positive to glucose fermentation test. All the 35 isolates failed to utilize fructose and sucrose. The result of the present study indicated that actinobacteria are highly non-specific in their carbon requirements. Strzelczyk (1981) also reported the non-specificity of actinobacteria for carbon requirements. The present study also revealed that the isolates varied in their response to catalase and oxidase. According to Dezfully and Ramanayaka (2015) *Streptomyces flavogriseus* ACTK2 was positive to catalase test and negative to oxidase

test. A study conducted by Vyawahare et al. (2013) also revealed that nine strains of *Streptomyces* were positive to both catalase and oxidase test.

Like other PGPR, actinobacteria also employ both direct and in-direct mechanisms to influence the plant growth and tolerance to stress conditions. The direct mechanisms involve production of indispensable factors for crop growth such as growth hormones like IAA, gibberellic acid, cytokinins *etc.* and help in nitrogen fixation, phosphate solubilization, and iron acquisition. PGP actinobacteria indirectly influence the plant growth by controlling and reducing the harmful effects of outside stresses of either biotic or abiotic sources through the following means: competition for nutrients, production of low molecular inhibitory substances such as ammonia, cyanogens, sulfides, aldehydes, alcohols and ketones, cell-wall degrading enzymes, and secondary metabolites with biocidal properties, in which the latter, two are the important event utilized by the actinobacterial community (El-Tarabily and Sivasithamparam 2006).

IAA is the principal form of auxin in plants and it plays an important role in regulating various physiological processes including cell division, elongation, differentiation and increase root hair formation (Khalid et al., 2004). Biosynthesis of IAA is considered very crucial in plant growth and development (Ali et al., 2009). Many actinobacteria isolated from the rhizosphere of different crops have greater capacity to produce IAA *in vitro*. Bacteria producing significant amount of IAA have been studied as bio-fertilizers (Naveed et al*.,* 2015).

In the present study, 35 actinobacterial isolates were screened for IAA production on the basis of development of pink colour in presence of tryptophan. IAA is a metabolite derived from Trp by many Trp-dependant and Trp-independent pathways in bacteria. More than one pathway could be present in a bacterium (Pattern and Glick, 1996). Physiological evidence for different Trp-dependent pathways for synthesis in *Azospirillum brasilense* was reported (Carreno-Lopez *et al.*, 2000). In Trp dependant pathway, tryptophan is converted to indole-3-acetamide (IAM) by tryptophan-2-monooxigenase and IAM is metabolized to IAA by IAM-hydrolase (Matsukawa *et al*., 2007). In the present investigation, all the 35 isolates were positive in the preliminary screening for IAA production. These isolates were classified into

four groups, based on the efficiency to produce IAA. Three isolates were grouped under the excellent category, one as good, 13 as moderate and 18 as poor producers of IAA. Quantitative estimation of IAA production under *in vitro* conditions was carried out. IAA production varied significantly with isolates from 0.43 μg $ml^{-1}$ to 15.9 μg $ml^{-1}$. Two isolates (Ptd-A and Amb-C) were found to be significantly superior to the other isolates, with IAA production of 15.9 μg $ml^{-1}$ and 15.38 μg $ml^{-1}$ respectively. Patten and Glick (1996) reported that 80 per cent of the microbes isolated from the rhizosphere of various crops have the capacity to produce indole acetic acid. It was reported that auxin production by plant growth promoting rhizobacteria can vary between different species, strains, microbial stage, availability of substrate and environment condition (Mirza et al., 2001). Suksaard et al. (2017) reported that 51% of the mangrove actinobacterial isolates produced IAA in the range of 0.21 μg $ml^{-1}$ to 165.74 μg $ml^{-1}$. Khamna et al. (2009) revealed that 81% of the isolates from the medicinal plants rhizosphere soil produced IAA in the range of 5.47 μg $ml^{-1}$ to 143.95 μg $ml^{-1}$. In a similar study conducted in wheat and tomato fields of Punjab, Pakistan, the most active (IAA) producer *Streptomyces nobilis* WA-3, *Streptomyces kunmingenesis* WC-3, and *Streptomyces enissocaesilis* TA-3 produced 79.5, 79.23, and 69.26 μg $ml^{-1}$ IAA respectively at 500 μg $ml^{-1}$ L-tryptophan (Anwar et al., 2016). Myo et al. (2019) optimised IAA production by *S. fradiae* NKZ-259 to explore formulations best suited for commercial use in agriculture. IAA production was elevated by optimising the culture conditions using a statistical design method and after conducting field experiments with tomato as the test crop, concluded that the strain was a promising and effective PGPR inoculant for plant growth promotion that may enrich soil fertility and enhance crop yields. Sameera et al. (2018) screened thirty two isolates of actinobacteria for IAA production and they found that 50 per cent of them belonged to *Streptomyces* species. *Streptomyces violaceolatus* was found to produce highest amount of IAA in ISP2 medium containing 0.5 per cent tryptophan. An endophytic actinobacterium (*Nocardiopsis*) associated with mandarin recorded highest IAA production of 222.75 μg $ml^{-1}$ (Shutsrirung et al. 2013)

Nitrogen is one of macronutrients essential for plant growth and productivity. Even though the atmosphere contains nitrogen to the extent of 78 %, it is unavailable

to the plants. The atmospheric nitrogen is converted into plant available forms by prokaryotic microorganisms. Atmospheric nitrogen is converted to plant-available form by these microorganisms, with the help of a nitrogenase enzyme complex system. This process is known as Biological Nitrogen Fixation (BNF) (Kim and Rees, 1994).

In the present study, thirty-five actinobacterial isolates were positive to qualitative screening for growth in N-free medium indicating possibility of being N-fixers. These isolates were grouped under different categories, based on the ability to grow on N-free medium, as excellent (seven isolates), good (16), moderate (10) and poor (two). Seven isolates which grew excellently well on N-free medium were further selected for assessing the amount of nitrogen fixed. Among these, four isolates *viz.* Ptd-A, Ptd-E, Ptd-B and Ptr-A recorded significantly superior nitrogen fixation of 24.7 mg (by Ptd-A and Ptd-E), 23.3 mg (Ptd-B) and 22.87 mg per gram of sucrose utilized (Ptr-A). It has been reported that many of the soil actinobacteria of the genera *Streptomyces, Frankia, Micromonospora, Nocardia etc*. have symbiotic as well as asymbiotic associations with plant organs, especially roots. There are numerous evidences of actinobacteria involved in nitrogen fixation. The genus *Frankia* are widespread endophytic actinobacteria symbiotically associated with plant roots and fix atmospheric nitrogen for host plants (Benson and Silvester 1993). In a report by Tokala et al. (2002), nitrogen fixation in roots of young pea seedlings with *Rhizobium* sp. was observed to be enhanced by *Streptomyces lydicus* strain WYEC108. Similarly, in the legume plants, the nodule inhabiting actinobacteria such as *Micromonospora*, *Streptomyces* sp., *Nocardia alba*, *Nonomuraea rubra*, and *Actinomadura glauciflava* have probiotic effects with *Rhizobium* (Swarnalakshmi et al., 2016). The diazotropic bacteria like *Microbacterium* and *Cellulosimicrobium* obtained from Wayanad were found to be fix high amount of nitrogen (Fathima, 2015).

Phosphorus is one of the major growth limiting macronutrients, second to nitrogen, for plants and accounts for around 0.2% of plant dry weight. It is present in soils as both organic and inorganic forms. Although large pool of P is available in soil, the quantity of forms available to plants is generally low, as the majority of soil P is found in the insoluble form. A large amount of phosphorous applied as fertilizers enters

into the immobile pools through precipitation reaction with highly reactive $Al^{3+}$ and $Fe^{3+}$ in acidic and $Ca^{2+}$ in calcareous or normal soils (Gyaneshwar et al., 2002). Organically bound phosphorous enters in the soil during the decay of natural vegetation, dead animals and from excretions. These organic forms of phosphorus are insoluble and hence remain unavailable to plants. Plants absorb phosphorus only in two soluble forms, as monobasic ($H_2PO_4^-$) and the dibasic ($HPO_4^{2-}$) ions (Bhattacharyya and Jha, 2012). It is widely accepted that soil microbes are involved in biological phenomena, including nitrogen fixation, phosphorus transformation and potassium solubilization in soil. These microbes solubilize insoluble phosphates like for plant growth (Rodriguez and Fraga, 1999).

In the present study, among 35 actinobacterial isolates, only six were positive screening, for phosphate solubilisation. Formation of clear zone on Pikovskaya's agar medium is considered to be indicative of phosphate solubilization. The study conducted by Li et al. (2019) revealed that among 43 bacterial isolates, thirteen showed no halo zone and thirty strains showed strong capacity for phosphorus solubilisation. Franco-correa and Chavarro-Anzola, (2016) obtained 33 actinobacterial isolates, among which 23 isolates showed clear halo zone, indicating phosphate solubilization efficiency. In the present investigation, estimation of phosphate solubilization revealed that the amount of phosphate solubilized was significantly higher in Ptd-E (40.74 μg $ml^{-1}$), Ptd-D (40.46 μg $ml^{-1}$), Ptr-E (40.09 μg $ml^{-1}$), Ptd-A (39.64 μg $ml^{-1}$) and Ptr-A (38.89 μg $ml^{-1}$), as compared to the isolate Ptd-B, which solubilized 31.55 μg $ml^{-1}$. Various genera of actinomycetes such as *Rhodococcus, Arthrobacter, Streptomyces, Gordonia and Micromonospora* were reported to have P-solubilization potential under laboratory and glasshouse conditions (Jog et al., 2014). Prada et al. (2014) isolated 57 strains of actinobacteria and estimated phosphorus solubilization ability, which ranged from 8.7 to 118.4 mg/kg and pH from 4.0 to 5.9.

Production of numerous organic acids such as gluconic acid, citric acid, malic acid, lactic acid, propionic acid, oxalic and succinic acids by actinomycetes are believed to be the process of their phosphate solubilisation, which also results in reduction of pH of the media (Hamdali et al., 2010). The quantity of P solubilized by microbes depends

on source of phosphorus and to a large extent by the growth conditions. Similar studies reviewed by Kpomblekou and Tabatabai (1994) pointed out that the microbes which lower the pH of the medium during the growth are efficient P solubilizers. In the present investigation also, a reduction in the pH of the medium was noticed in all the six isolates, and the lowest pH was recorded by Ptd-E and Ptd-D, which also solubilized phosphate efficiently.

Potassium is the third major essential macronutrient required for plant growth. The soluble K concentration in soil is low and more than 90% of it exists in the form of insoluble rocks and silicate minerals. Rhizosphere microbes have the capacity to dissolve potassium from insoluble minerals in soil and form exchangeable total soil K pools by chelation, acidolysis and solubilization (Uroz et al., 2009). In the present study, none of the isolates exhibited the ability for potassium solubilization on Aleksandrov agar. However, in a study conducted by Nafis et al. (2019), of the 23 isolates of actinobacteria, only one isolate was unable to form a clear zone, sixteen isolates produced less clear zone while six isolates showed relatively large clear zone on modified Aleksandrov agar plates. Wang et al. (2020) isolated 39 PGPRs, 13 isolates were nitrogen fixers, 11 isolates were efficient phosphate solubilizers, 15 were potassium solubilizers and 1 was 1AA producer. Nafis et al. (2019) reported that currently, little information is available on potassium solubilization by rhizospheric actinobacteria.

Zinc is an essential micronutrient required for plant growth and development. The quantity of Zn applied in inorganic form is converted into unavailable form in soil. Zinc solubilizing bacteria are reported as potential substitutes for zinc additive (Praveen et al., 2013). They can solubilize the insoluble form into soluble form of zinc and make it available to the plant. None of isolates in the present investigation were zinc solubilizers. Patel and Thakker (2020) reported zinc mineralization by a strain YM4, with a solubilisation index of 76.31. Yaghoubi et al. (2018) conducted an experiment to evaluate the zinc solubilizing ability of 80 PGPB strains isolated from the rhizosphere of barley and tomato plants. Among 80 isolates, only two strains (*Agrobacterium tumefaciens* and *Rhizobium* sp.) produced clear halo zone around their colonies in solid medium supplemented with zinc oxide 0.1%.

In the present investigation, potassium solubilizing actinobacteria were not obtained from Wayanad soils. This could be related to the availability of these two nutrients in sufficient levels in the soil. This requires further analysis of the K and Zn content in natural soils of Wayanad.

Eleven isolates of actinobacteria were selected based on their IAA production. These were ranked based on their efficiency to promote plant growth through IAA production, N-fixation and phosphate solubilization. Three isolates with one to three were selected as the most efficient ones. These isolates were further identified by 16S rRNA gene sequencing. The selected actinobacterial isolates were Ptd-E, Ptd-A (both obtained from Puttad location), and Ptr-A isolated from Pathiri.

Three selected isolates were further subjected to molecular characterization by 16S rRNA gene sequencing by the Sanger's method. Woese and others, reported that phylogenetic relationships of bacteria, and, indeed, all life-forms, could be determined by comparing a stable part of the genetic code (Woese, 1985). Woese (1985) reported that 16S rRNA gene is the best molecular chronometer with greater resolving power than other oligonucleotides used earlier for cataloguing purposes. The part of the DNA most commonly used for taxonomic purposes in bacteria is the 16S rRNA gene, which exists universally among bacteria. This gene includes regions with species-specific variability, which makes it possible to identify bacteria to the genus or species level by comparison with databases in the public domain (Clarridge, 2004). The conserved regions are used for designing primers, which will amplify the gene. In this study primers 16S-RS-R and 16S-RS-F were used to amplify the 1500bp 16S rRNA gene. Molecular characterization provides added advantage that it is not affected by environmental factors or nutrient composition of the medium, unlike phenotypic characters. Therefore, 16S rRNA gene sequences allow bacterial identification that is more strong, reproducible, and precise than that obtained by phenotypic testing. The sequences obtained from the three isolates were analysed using BLASTn, to find out the similarity with sequences available in NCBI databank. GenBank (www.ncbi.nlm.nih.gov/genbank) is a comprehensive database that contains publicly available nucleotide sequences for 3,70,000 formally described species and daily data exchange with the European Nucleotide Archive (ENA) and the DNA data Bank of

Japan (DDBJ) ensure worldwide coverage (Benson et al., 2017). The accession sharing maximum homology with the query sequence was considered for identification of the isolate. The three selected isolates were identified as *Streptomyces* sp. (Ptd-A), *Actinobacteria bacterium* Ptd-E) and *Streptomyces* sp. (Ptr-A). 16S rRNA gene sequences of three isolates were submitted to the Gene Bank of NCBI, to get accession numbers. The tentative names assigned to each isolate based on the location was retained as strain number.

Among the several genera of actinomycetes, *Streptomyces* are classified as most abundantly occurring actinomycete in the soil. *Streptomyces* has gained importance in numerous sectors like health and agriculture, and it is also considered as most dominant actinobacteria, for root colonization and close association with plant roots. N-fixation, phosphate solubilization, IAA production, siderophore production and ACC deaminase activity have been reported in various species of Streptomyces (Rungin et al., 2012; Aly et al., 2012). Several studies have indicated that *Streptomyces* can produce abundant metabolites, which play various roles in agriculture such as plant–promoting and against phytopathogens (Kaur et al., 2019).

Phylogenetic analysis was carried out for assessing the evolutionary relationships of the three isolates used in the present study, with already reported strains of relevant species. Phylogenetic trees were constructed using MEGA 7 software. The phylogenetic tree showed two major clusters. Cluster 1 had two sub-clusters: sub-cluster 1 contained *Streptomyces* sp. strain Ptd-A and *Actinobacteria bacterium* strain Ptd-E together with 91 per cent bootstrap confidence value and Sub-cluster 2 consisted of *Streptomyces* sp. strain Ptr-A was closely related to *Streptomyces* sp. strain MJM16245, *Streptomyces* sp. strain WI04-3A and *Streptomyces* sp. strain HF-6 with a bootstrap confidence value of 100.

After *in vitro* screening of isolates, the plant growth promoting activities were evaluated under *in vivo* conditions, in sterile soil in with black pepper (variety Panniyur-1) as the test crop. The potting mixture was sterilized by treatment with formaldehyde. Soil sterilization by fumigation with formaldehyde has been practiced since a long time. Warcup (1951) reported that fungi were destroyed or reduced to low numbers by steam formalin treatment.

The treatments included the three most efficient isolates (Ptd-E, Ptd-A and Ptr-A), the fourth treatment comprised PGPR mix-1 (commercial formulation of KAU). Organic Package of Practices Recommendations (2017) were used for comparison with the microbial inoculants. Actinobacteria were made into talc-based formulation and applied at the time of planting and 45 days after planting to soil. According to Gopalakrishnan et al. (2016), the efficacy of microbial inoculants largely depends on the type of formulation and the delivery technology that extends the shelf lives for at least few months and in all cases the PGP/antagonistic activity should be retained. The production cost also has to be kept to a minimal while developing a microbial formulation. A good formulation should be easy to handle and apply so that it is delivered at the target site and protects the PGP microbes and enhances its activity from harmful environmental factors under field conditions. Talc is one of the common means of application of bacterial inoculants to soil composed of minerals in combination with chloride and carbonate and referred as steatite or soapstone or magnesium silicate (Nakkeeran et al. 2005). Talc has also been used in the preparation of fungal and actinobacterial bioinoculants. Talc-based formulation of *Streptomyces griseus* , either as single or with chitin, was demonstrated to have stable shelf life of up to 105 days and control *Fusarium oxysporum* f. sp. *lycopersici* , which causes Fusarium wilt in tomato (Anitha and Rabeeth, 2009 ). Tamreiho and Nongthomba (2016) reported roots of 10 rice plants were dipped in one g talc powder of *Streptomyces corchorusii* strain UCR3-16 under net house conditions and the roots were dipped in freshly prepared talc powder formulation under field conditions.

The population of actinobacteria in talc-based formulation was assessed one week before application by serial dilution and plating technique. The population of actinobacteria in three formulations were found to be $10^8$ cfu $g^{-1}$.

In the *in-planta* experiment, biometric characters were recorded at monthly intervals, up to five months. The actinobacterial treatment, $T_1$: Ptd-E (*Actinobacteria bacterium*), $T_2$: Ptd-A (*Streptomyces* sp.) and $T_3$: Ptr-A (*Streptomyces* sp.) showed significant increase in shoot length, number of leaves and internode length throughout the growth period from planting to five month after planting.

Significantly higher root growth was observed in treatment $T_2$: Ptd-A (*Streptomyces* sp.), with significantly higher root volume, fresh and root weight. This isolate was found to be the most efficient IAA producer and good phosphate solubilizer also. Many studies revealed that combination of IAA production and phosphate solubilization by microbes in soil promote plant growth. Barbieri and Galli (1993) reported that most rhizosphere bacteria produce IAA and they enhanced the lateral and adventitious roots formation. Understanding and quantifying the impact of PGPR on roots has been a major challenge to scientists. *In-vitro* evaluation by inoculating the roots with PGPR has revealed that many PGPR may reduce the growth rate of the primary root (Dobbelaere et al., 1999), increase the number and length of lateral roots (Combes-Meynet et al., 2011; Chamam et al., 2013), and stimulate root hair elongation *in vitro* (Dobbelaere et al., 1999; Contesto et al., 2008). As a result, the uptake of minerals and water, and thus the growth of the whole plant, will be increased. Enhanced root and shoot biomass have been documented for PGPR-inoculated plants growing in soil (El Zemrany et al., 2006; Walker et al., 2012). Similarly, phytohormone producing capacity of several rhizospheric and endophytic actinobacteria has also been documented by researchers for IAA, cytokinins, and gibberellins (El-Tarabily and Sivasithamparam 2006; Vijayabharathi et al. 2016). Endophytic actinobacteria are also drawing great interest. *Nocardiopsis*, an endophytic actinobacterium in mandarin recorded highest IAA production (222.75 ppm) (Shutsrirung et al. 2013). IAA producing endophytic *Streptomyces atrovirens, Streptomyces olivaceoviridis, Streptomyces rimosus, Streptomyces rochei,* and *Streptomyces viridis* resulted in improved root elongation and growth (El-Tarabily 2008; Khamna et al. 2010; Abd-Alla et al. 2013). IAA also induced cell differentiation, hyphal elongation, and sporulation in *Streptomyces atroolivaceus* (Matsukawa et al. 2007). Metabolites like pteridic acids A and B produced by endophytic *Streptomyces hygroscopicus* were found to show auxin-like activity and induce root elongation in common bean (Igarashi et al. 2002). Hence, the phytohormone producing potential and metabolites with phytohormone mimicking activity of actinobacteria can be exploited for enhancing crop productivity.

The work conducted at the Department of Agricultural Microbiology (KAU) under the All-India Network Project on soil biodiversity and biofertilizers, actinobacteria belonging to two genera *i.e., Microbacterium* and *Cellulosimicrobium* were found to be fix high amounts of nitrogen. Therefore, they were developed into talc-based formulations and experiments were conducted in black pepper as well as ginger. They were found to improve growth and yield in black pepper and ginger. Thampi and Bhai (2017) conducted *in planta* evaluation for plant growth promotion and biocontrol efficacy by rhizosphere actinobacteria in black pepper (*Piper nigrum* L.). Out of three isolates, IISRBPAct1 showed remarkable increase in fresh shoot weight, shoot height and number of nodes. Another important character observed for IISRBPAct1 was the early formation of laterals, which is notably high as compared to *Trichoderma* treatment and control plants. However, a huge and vast root system was observed in treatment with IISRBPAct-42 and so maximum increase in fresh and dry root biomass was observed in this treatment. The identified three potential *Streptomyces* spp., *i. e*., IISRBPAct-1, IISRBP Act-25 and IISRBPAct-42 were very effective in controlling the pathogens under *in vitro* and *in planta* conditions. *Streptomyces* sp. strain DBT204 isolated from tomato showed plant growth promoting traits in seedlings of both chilli and tomato by Passari et al. (2016). The two potential strains *Streptomyces* sp. (BPSAC34) and *Leifsonia xyli* (BPSAC24) were identified by Passari et al. (2015) with plant growth promotion and when tested *in vivo* showed enhanced a range of growth parameters in chilli (*Capsicum annuum* L.) under greenhouse conditions.

Among three most potential plant growth promoting actinobacteria were selected, all actinobacterial isolates are efficient for promoting plant growth. These isolates may further be evaluated under field conditions for plant growth promotion, disease suppression and drought tolerance ability. The compatibility of the actinobacteria with other plant growth promoting microorganisms and chemical fertilizers may also be evaluated. In addition, expansion of local production models for commercialization of these microbial inoculants is also essential.

Actinobacteria have numerous potential benefits, they can survive for longer periods in soil, in the form of spores, which may be an added advantage to develop as

a biofertilizers. They are a good source of novel antibiotics, anti-cancer agents, anti-fungals, and other secondary metabolites that can be used in medicine or to improve plant growth and disease resistance. Actinobacteria are also very promising tool for biocontrol of pests and as plant growth promoters. The isolates having multiple plant growth promoting traits can be exploited for development of consortium. Actinobacteria are capable of degrading hydrocarbons, pesticides, fungicides *etc.* and their metabolic potential offers a good area of research. The native actinobacterial isolates can be used for enhancing crop production as well as soil fertility in an eco-friendly manner. Only little information is available on potassium and zinc solubilizing actinobacteria and therefore further studies are required in this field. Many of the actinobacterial genera have not been exploited for agricultural, biotechnological or industrial purposes. Hence, actinobacterial diversity and the biochemical molecules produced by actinobacteria could be exploited to address the issues to build sustainable and eco-friendly agriculture.

# 6. *Summary*

# 6. SUMMARY

The study entitled "Plant growth promoting actinobacteria from rhizosphere soils of black pepper in Wayanad" comprising both laboratory and *in planta* experiments were conducted in Department of Agricultural Microbiology, College of Agriculture, Vellanikkara, during 2018- 2021. The main objective of the study was to assess native actinobacteria from black pepper rhizosphere soils of flood affected and non- flood affected areas of Wayanad with respect to plant growth promotion. The important findings of the study are summarized below:

- Ten rhizosphere soil samples were collected comprising five each from non-flood affected and flood affected black pepper growing areas of Wayanad district.
- Population of actinobacteria was enumerated on three different media- Actinomycetes Isolation Agar, Kenknight & Munaier's Agar and Starch Casein Agar.
- The location Puttad recorded significantly higher actinobacterial population on all the three media
- No actinobacterial colonies were detected in any of the flooded soil samples on any medium, even at a dilution of $10^{-1}$, except in Meppadi soil
- Among the different media, Starch Casein Agar recorded higher population in all the non-flooded locations, except Pulpally.
- A total of 35 isolates (34 from non-flooded areas and one from flood affected area) were purified and maintained in Kenknight & Munaier's agar.
- Cultural and morphological characteristics of selected isolates were studied on Kenknight & Munaier's agar media.
- Spore chain morphology varied among genera and included rectiflexibiles, spira, short chain and closed spira
- All the isolates were Gram positive in nature.
- Biochemical characterization (catalase test, oxidase test and utilization of sugars) of selected isolates were studied.
- Among 35 isolates, 26 isolates were positive to catalase test, 13 isolates were positive to oxidase test, only 3 isolates were positive to glucose fermentation test and all isolates were negative to fructose and sucrose fermentation test.

- All the 35 isolates were screened for plant growth promoting (PGP) activities *in vitro*.
- The isolates Ptd-A and Amb-C were found to be significantly superior to all other isolates, with IAA production of 15.9 μg $ml^{-1}$ and 15.38 μg $ml^{-1}$ respectively.
- Four isolates *viz.* Ptd-A, Ptd-E, Ptd-B and Ptr-A recorded significantly superior nitrogen fixation of 24.7 mg (Ptd-A and Ptd-E), 23.3 mg (Ptd-B) and 22.87 mg (Ptr-A) per gram of sucrose source provided in the medium.
- Estimation of solubilized P by the phosphomolybdic blue colour method revealed significantly higher solubilization in Ptd-E (40.74 μg $ml^{-1}$), Ptd-D (40.46 μg $ml^{-1}$), Ptr-E (40.09 μg $ml^{-1}$), Ptd-A (39.64 μg $ml^{-1}$) and Ptr-A (38.89 μg $ml^{-1}$).
- None of the isolates solubilized potassium and zinc.
- Based on PGP activities, three predominant isolates were selected for *in planta* experiment.
- The selected isolates were further identified by 16S rRNA gene sequence analysis. The test isolates were identified by the accession in NCBI database and showing maximum homology with query sequence.
- Phylogenetic tree constructed using MEGA 7 software two clusters. In first cluster, the actinobacterial isolate *Streptomyces* sp. strain Ptd-A and *Actinobacteria bacterium* strain Ptd-E were grouped together. In the second cluster, *Streptomyces* sp. strain Ptr-A was closely positioned to *Streptomyces* sp. strain MJM16245, *Streptomyces* sp. strain WI04-3A and *Streptomyces* sp. strain HF-6.
- The isolates Ptd-A and Ptr-A were identified as *Streptomyces* sp. and Ptd-E as *Actinobacteria bacterium*.
- The selected isolates were evaluated for their plant growth promotion activities in black pepper variety Panniyur-1 and PGPR mix-1 and Organic Package of Practices Recommendations (2017) were used for comparison.
- The native actinobacterial isolates, $T_1$: Ptd-E (*Actinobacteria bacterium*), $T_2$: Ptd-A (*Streptomyces* sp.) and $T_3$: Ptr-A (*Streptomyces* sp.) showed significant increase in shoot length, number of leaves and internode length throughout the growth period from planting to five MAP.
- The higher root growth was observed in treatment $T_2$: Ptd-A (*Streptomyces* sp.), with significantly higher root volume, fresh and dry weight of roots.

- Significantly higher plant fresh weight was observed in treatment $T_2$: Ptd-A (*Streptomyces* sp.) and $T_2$: Ptd-A (*Streptomyces* sp.) and $T_3$: Ptr-A (*Streptomyces* sp.) showed significant higher plant dry weight.
- The study indicated the possibility of exploitation of actinobacteria with multiple plant growth promoting activities for improving the growth of black pepper in Wayanad.

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# *Appendices*

## APPENDIX- I

## MEDIA USED AND COMPOSITION

### a) Kenknight and Munaier's media

| | |
|---|---|
| Dextrose | : 1 g |
| $K_2HPO_4$ | : 0.1 g |
| $NH_4SO_4$ | : 0.1 g |
| KCl | : 0.1 g |
| $MgSO_4$ | : 0.1 g |
| Agar | : 20 g |
| Distilled water | : 1000 ml |
| pH | : 7 |

### b) Starch Casein Agar

| | |
|---|---|
| Soluble starch | : 10 g |
| Vitamin free Casamino acids | : 0.3 g |
| $CaCO_3$ | : 0.02 g |
| $Fe_3SO_4.7H_2O$ | : 0.01 g |
| $MgSO_4.7H_2O$ | : 0.05 g |
| $KNO_3$ | : 2 g |
| NaCl | : 5 g |
| Agar | : 18 g |
| Distilled water | : 1000 ml |
| pH | : 7.1±0.1 |

### c) Actinomycetes Isolation Agar

| | |
|---|---|
| Sodium caseinate | : 2 g |
| L-Asparagine | : 0.1 g |
| Sodium propionate | : 4 g |
| Dipotassium phosphate | : 0.5 g |

| | |
|---|---|
| $MgSO_4.7H_2O$ | : 0.1 g |
| $Fe_3SO_4.7H_2O$ | : 0.001 g |
| Agar | : 15 g |
| Distilled water | : 1000 ml |
| pH | : 8.1±0.2 |

**d) Mineral Salt Agar amended with 0.1% ZnO**

| | |
|---|---|
| $NaNO_3$ | : 2 g |
| $Fe_2(SO_4)_3. H_2O$ | : 0.01 g |
| KCl | : 0.5 g |
| $KH_2PO_4$ | : 0.14 g |
| $MgSO_4.7H_2O$ | : 0.5 g |
| $K_2HPO_4$ | : 1.2 g |
| Yeast extract | : 0.02 g |
| ZnO | : 1 g |
| Agar | : 15 g |
| Distilled water | : 1000 ml |
| pH | : 7.2±0.1 |

**e) Jensen's agar**

| | |
|---|---|
| Sucrose | : 20 g |
| Dipotassium phosphate | : 1 g |
| Magnesium sulphate | : 0.5 g |
| Sodium chloride | : 0.5 g |
| Ferrous sulphate | : 0.10 g |
| Sodium molybdate | : 0.005 g |
| Calcium carbonate | : 2 g |
| Agar | : 20 g |
| Distilled water | : 1000 ml |

**f) Pikovskaya's agar**

| | |
|---|---|
| Glucose | : 10 g |
| Tri calcium phosphate | : 5 g |
| Ammonium sulphate | : 0.5 g |
| Sodium chloride | : 0.2 g |
| Magnesium sulphate | : 0.10 g |
| Potassium chloride | : 0.2 g |
| Yeast extract | : 0.50 g |
| Manganese Sulphate | : 0.002 g |
| Ferrous Sulphate | : 0.002 g |
| Distilled water | : 1000 ml |
| pH | : 7.00 |

**g) Aleksandrov's agar**

| | |
|---|---|
| Glucose | : 5 g |
| Calcium phosphate | : 2 g |
| Calcium carbonate | : 0.10 g |
| Ferric chloride | : 0.005 g |
| Magnesium sulphate | : 0.50 g |
| Potassium aluminosilicate | : 2 g |
| Distilled water | : 1000 ml |
| pH | : 7.2 |

## APPENDIX- II

## REAGENTS USED

**a)** Ammonium molybdate reagent

12g of Ammonium molybdate is dissolved in 250 ml of distilled water. 0.291g of antimony potassium tartarate is dissolved in 100 ml of distilled water. Both these solutions are added to 1000 ml of approx. 5 N $H_2SO_4$. This solution is mixed thoroughly and made up to 2L with distilled water.

**b)** Boric acid indicator mixture

0.2% Bromocresol green + 0.2 % Methyl Red in alcohol in 5:1 ratio

**c)** Salkowski reagent

2% of 0.5 M $FeCl_3$ in 35% Perchloric acid

# PLANT GROWTH PROMOTING ACTINOBACTERIA FROM RHIZOSPHERE SOILS OF BLACK PEPPER IN WAYANAD

**By**

**RINEESHA BACKER A.**

**(2018-11-091)**

**ABSTRACT OF THE THESIS**

***Submitted in partial fulfilment of the requirement for the degree of***

## Master of Science in Agriculture

**Faculty of Agriculture**

**Kerala Agricultural University**


**DEPARTMENT OF AGRICULTURAL MICROBIOLOGY**

**COLLEGE OF AGRICULTURE**

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# ABSTRACT

Black pepper (*Piper nigrum* L.) known as the 'King of spices' is one of the important export-oriented spice crops that provides major source of income and employment for rural households in Kerala. Wayanad is one of the main pepper growing tracts in Kerala. However, the production of black pepper in Wayanad has been declining over the past many years, mainly due to the poor soil health status, improper land management and changes in climatic factors. An increasing demand for low-input agriculture has resulted in a greater interest in soil microorganisms which are able to enhance plant health and soil quality. However, actinobacteria have not yet been exploited for this purpose. Hence the present study focused on isolation, screening and characterization of actinobacteria from rhizosphere soils of black pepper in Wayanad and evaluation of their plant growth promoting activity.

Rhizosphere soil samples were collected from five different locations of flood affected and non-flood affected black pepper growing areas of Wayanad district. Enumeration of actinobacteria on three different media revealed that the population of actinobacteria ranged from $1.00 \times 10^4$ cfu to $12.00 \times 10^4$ cfu $g^{-1}$ soil in non-flooded soil. Actinobacteria could not be detected in flooded soils, even at a dilution of $10^{-1}$, except in Meppadi. Among the different media, starch casein agar recorded higher population of actinobacteria. A total of 35 isolates were purified and maintained for characterization and screening for plant growth promoting activities.

Cultural, morphological and biochemical characters of all 35 isolates of actinobacteria were studied. All the isolates were Gram positive, and they varied in the colony morphology, colour of aerial mycelium, spore chain morphology and pigmentation. Two isolates Vlt-K and Plp-B produced yellow water-soluble pigment and Vlt-I produce purple water insoluble pigment.

Screening of all the 35 isolates under *in vitro* conditions for plant growth promoting (PGP) activities revealed that the isolates were highly variable. Two isolates (Ptd-A and Amb-C) were superior to other isolates in IAA production. Significantly higher N-fixation was noticed in four isolates (Ptd-A, Ptd-E, Ptd-B and Ptr-A). Six isolates solubilized insoluble phosphate to available P, and also reduced the pH of the

medium. Reduction in pH by phosphate solubilizers has been reported earlier and this is attributed to the production of organic and inorganic acids. None of the isolates solubilized K or Zn.

Three isolates with multiple PGP activities were selected, based on ranking for PGP activities *in vitro* and identified by 16S rRNA gene sequencing (Ptd-A and Ptr-A as *Streptomyces* sp. and Ptd-E as *Actinobacteria bacterium*). These actinobacterial isolates evaluated *in-planta* for PGP activities with black pepper cuttings (variety Panniyur-1). Treatments also included PGPR mix-1 (KAU commercial formulation) and Organic Package of Practices Recommendations (2017). All the three native isolates exhibited significant increase in shoot length, number of leaves and internode length throughout the growth period from planting to five months. Significantly higher root growth was observed in treatment $T_2$: Ptd-A (*Streptomyces* sp.), with significantly higher root volume, fresh and dry root weight. Significantly higher plant fresh weight was observed in $T_2$: Ptd-A (*Streptomyces* sp.) and significantly higher plant dry weight was observed in $T_2$: Ptd-A (*Streptomyces* sp.) and $T_3$: Ptr-A (*Streptomyces* sp).

The present investigation revealed that native actinobacteria have the potential to improve the growth of black pepper. These isolates may further be evaluated under field conditions before commercialization. Screening for other beneficial traits like disease suppression and drought tolerance may also be carried out. The compatibility of the actinobacteria with other PGP microorganisms may also be evaluated. Actinobacterial spores survive in soil for longer periods and this may be an added advantage. Actinobacterial diversity and the biochemical molecules produced by actinobacteria could be exploited to build up sustainable and eco-friendly agriculture.