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FUNCTIONAL DIVERSITY OF BENEFICIAL MICROORGANISMS FROM THE RHIZOSPHERE OF BLACK PEPPER IN WAYANAD

By

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THESIS

Submitted in partial fulfilment

of the requirement for the degree of

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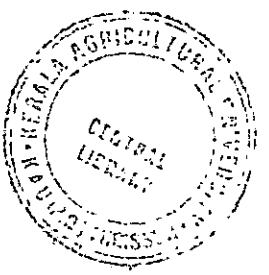

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DECLARATION

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

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Dedication…

To my father

His words of inspiration an

encouragement

in pursuit of excellence, still linger on…

ABBREVIATIONS

ARDRA	Amplified ribosomal DNA restriction analysis
BNF	Biological nitrogen fixation
bp	Base pair
BV	Blank value
CAS	Chrome azurol S
CRD	Complete randomized design
DNA	Deoxyribo Nucleic Acid
DRB	Deleterious rhizobacteria
cm	Centi metre
cfu	Colony forming unit
g	Grams
g^{-l}	Grams per litre
h	Hour(s)
ha	Hectre
HCN	Hydrogen cyanide
HCL	Hydrochloric acid
H_2SO_4	Sulphuric acid

kb	Kilo base
kg	Kilogram
MSL	Mean sea level
NCBI	National Center for Biotechnology Information
NCFT	National Center for Fungal Taxonomy
nm	Nano metre
m	Meter
mg	Milligram
min	Minutes
ml	Milliliter
mm	Millimeter
M	Molar
N	Normality
NaOH	Sodium hydroxide
PCR	Polymerase chain reaction
PGP	Plant growth promotion
PGPM	Plant growth promoting microorganisms
pH	Hydrogen ion concentration
PR	Pathogenesis related proteins

PSM	Phosphate solubilising microorganisms
rpm	Revolutions per minute
RKN	Root knot nematode
IAA	Indole acetic acid
IHR	Indian Himalayan region
ISR	Induced systemic resistance
SDS	Sodium Dodecyl Sulphate
TAE	Tris acetate EDTA buffer
Trp	Trptophan
TV	Titre value
UV	Ultraviolet rays
β	Beta
µg	Micro gram
µl	Micro litre
µM	Micro molar

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Introduction

1. INTRODUCTION

Black pepper is an important foreign exchange earning spice crop of Kerala. It is rightly called 'black gold' and 'King of Spices'. It is a woody climber and is a native of the Western Ghats of South India. The cultivation of black pepper is mainly confined to India, Brazil, Indonesia, Malaysia, Thailand, Sri Lanka and Vietnam. India is one of the largest exporters of pepper. In India black pepper is being cultivated in Kerala (96%), Karnataka (3%) and to a lesser extent, in Maharashtra, Andhra Pradesh, Tamil Nadu and north eastern regions.

Indian black pepper is preferred in the international market due to its proper combination of pleasant flavour, taste, piperine content and essential oil. It is not only used as a condiment but also, widely used in culinary preparations, food processing, perfumery and as an important ingredient in most of the Ayurvedic medicine preparations. The distribution pattern of pepper across various states of India showed the dominance of Kerala with 94.31%. Kerala contributes 96 per cent of area and 97 per cent of the total production in India.

Wayanad is an important pepper growing district of Kerala with an area of 36,488ha and a production of 9828 tonnes (FIB, 2009). Pepper is affected by several diseases and pests. *Phytophthora* foot rot and pepper yellows are serious problems in the pepper growing belts of Wayanad and Idukki. Since the pathogen is soil borne, native antagonistic microorganisms play a major role in keeping the population of pathogen at low levels. Though work on screening of some of the commercially available biocontrol agents for their antagonistic effect on *P. capsici* is available, not many reports on isolation, identification and studies on antagonistic ability of native. Even though specific control measures are available for foot rot, the exact cause of pepper yellowing is unknown. Yellowing can be caused by several factors including latent infection of pathogens like *Phytophthora, Fusarium*, plant parasitic nematodes, nutrient imbalance and by attack of mealy bugs etc.

Microbial diversity in soil ecosystems exceeds, by far that of eukaryotic organisms. One gram of soil may harbour upto 10 billion microorganisms of possibly thousands of different species (Rosello and Amann, 2001). The complexity of the soil system is determined by the numerous and diverse interactions among its physical, chemical, and biological components, as modulated by the prevalent environmental conditions (Buscot, 2005). Many studies have demonstrated that soil-borne microbes interact with plant roots and soil constituents at the root-soil interface (Barea *et al.*, 2005). The great array of root- microbe interactions results in the development of a dynamic environment known as the rhizosphere where microbial communities also interact.

Plant rhizosphere harbours extremely complex community including saprophytes, epiphytes, pathogens and beneficial microbes. The beneficial group can be classified into two broad groups based on their primary effects, plant growth promoting microorganisms (PGPM) and biological control agents. Rhizosphere microorganisms inhabit plant roots and exert a positive effect ranging from direct influence mechanisms to an indirect effect and are termed PGPM. PGPM are known to participate in many important ecosystem processes, such as the biological control of plant pathogens, nutrient cycling and seedling growth (Persello *et. al.*, 2003, Barea *et al.*, 2004, Zahir *et al.*, 2004). The production of phytohormones, which are signal molecules acting as chemical messengers by PGPM is now considered to be one of the most important mechanisms by which many rhizosphere microbes promote plant growth.

Conventional strategies of disease control were replaced with the use of chemicals. However, these chemicals affected soil fertility and the ecosystem. With root rot and spot, the efficacy of fungicides and plant genetic resistance is determined by the interaction of environmental and cultural conditions. To overcome this problem, biocontrol agents have been and are being, investigated.

Presently microbial antagonists are increasingly being used as plant growth promoters and for the management of plant diseases including those caused by soil-borne plant pathogens (Cook and Baker, 1983). Development of management strategies against diseases would help to regain India's glory as a major exporter of spices. Biological control has become a thrust area of research which in turn would minimize pesticide residues and environmental problems. Therefore it is necessary to develop efficient strains in field conditions. One possible approach is to explore soil microbial diversity for PGPM having combinaton of growth promotion and biocontrol activities well adapted to particular soil environment.

With this background, the present investigation was taken up with the following objectives.

- Isolation of rhizosphere microflora from pepper vines from healthy and diseased gardens of Wayanad
- Screening for plant growth promoting activities *in vitro*
- Screening for antagonistic activities against soil borne pathogen *in vitro*
- *In planta* screening of selected isolates for plant growth promotion and antagonistic activities

Review of literature

2. REVIEW OF LITERATURE

Black pepper (*Piper nigrum* L.) is a perennial, woody and flowering climber belonging to family Piperaceae. It is one of the important crops which provides major source of income and employment for rural households in Kerala, where more than 2.5 lakhs farm families are involved in pepper cultivation (Government of India, 2009). From time immemorial India has always reigned supreme in the production and export of this most renowned spice in the world. However, disease infestation in gardens is still the biggest problem in black pepper cultivation. Chemical control of these diseases has been practiced for several years. However, recently there is a great concern among scientists and farmers about the possibility of environmental pollution caused by these chemicals. Biological control is a promising alternative to control plant pathogens. Use of plant growth promoting microorganisms (PGPM) offers an attractive way to replace chemical fertilizer, pesticides and supplements and application of the same results in a significant increase in plant height, root length and dry matter production. Therefore, much research has been conducted on biological control agents effective in disease management.

Several attempts have been made to study the plant growth promotion and the management of *Phytophthora* disease in black pepper by chemical, physical and biological means. In this chapter, an attempt has been made to review the relevance and importance of these aspects, particularly on plant growth promotion and biocontrol of *P. capsici*.

2.1. AREA AND DISTRIBUTION OF BLACK PEPPER (*Piper nigrum* L.)

Black pepper, one of the most ancient spice crops cultivated in India, probably originated in the hills of South-Western India *i.e.* from North Kanara to Kanyakumari. It is mostly found in hot and moist parts of southern India. The distribution pattern of pepper across various states of India showed the dominance of

Kerala with 89 per cent of the total area under cultivation. Kerala alone contributes about 96 per cent of the total production in India, next comes Karnataka with 3.5 percent. The rest is contributed by Tamil Nadu, Pondicherry and Andaman and Nicobar.

In Kerala, Wayanad district dominated pepper farming in the country 20 years ago, producing more than 40,000 tonnes of the crop every year in the mid-1980s *i.e.,* about half of India's total pepper production. The variety grown in the district is the aromatic Tellicherry bold pepper, for which the Spices Board of India is attempting to get a geographical indication status, to acknowledge its unique pungent flavour. But the situation turned dramatically in 1990s, with the spread of the foot rot disease among Wayanad's pepper vines. According to data available with the state directorate of economics and statistics, Wayanad which produced 22,385 tonnes of pepper from 43,039 ha in 1995-96, harvested just 9,828 tonnes from 36,488 ha in 2006-07.

In Karnataka, Kodagu, North Kanara, South Kanara and Shimoga are major centers of pepper production. Likewise in Tamil Nadu, Kanyakumari, Nilgiris and Mahe in Pondicherry are the other major centers of pepper production. Black pepper is also grown in some parts of Orissa, Andhra Pradesh and North Eastern region. But now pepper cultivation has spread to some parts of Goa and Maharashtra also.

2.2. CLIMATE AND SOIL

Black pepper grows successfully between 20^0 North and 20^0 South of equator and from sea level upto 1500 m above MSL. It is a plant of humid tropics, requiring 2000 - 3000 mm of rainfall, tropical temperature and high relative humidity with little variation in day length throughout the year. Black pepper does not tolerate excessive heat and dryness. In India, black pepper growing areas receive 1500 mm to more than 4000 mm rainfall. Rainfall after stress induces profuse flowering (Pillay *et. al.,* 1955).

Soils suitable for black pepper at different production centers have been reviewed earlier (Waard, 1969; Purseglove *et al.,* 1981). It can be grown on various kinds of soils such as clay loam, red loam, sandy loam and laterite soils. Wardani and Zaubin (1984) reported that black pepper varieties differ in their growth with respect to soil pH and pH above 7.5 inhibits growth. However, Sangakkara (1989) reported that growth of the variety Panniyur 1 was highest at pH 7.8 and 8.1. Well drained loamy soils rich in humus nourish the crop well and the best crop could be obtained in virgin forest soil.

2.3. FUNCTIONAL DIVERSITY OF RHIZOSPHERE MICROFLORA

Functional diversity is an aspect of the overall microbial diversity in soil, and encompasses a range of activities. Microbial diversity is usually taken as the number of individuals assigned to different taxa and their distribution among taxa (Atlas and Bartha, 1998). The relationship between microbial diversity and function in soil is largely unknown, but biodiversity has been assumed to influence ecosystem stability, productivity and resilience towards stress and disturbance. Microbial activity in the rhizosphere is essential for plant functioning, as it assists the plant in nutrient uptake and offers protection against pathogen attack (Berendsen *et al.,* 2012).

Changes in the composition of soil microflora can be crucial for the functional integrity of soil (Insam, 2001). Nannipieri *et al.* (2003) has reviewed the inter relationship between the microflora, its diversity and function in soil. Microbial diversity is one of the key issues when the theme of soil emergency is discussed due to the role of structural and functional diversity of soil microorganisms in soil fertility, productivity and ecological stability.

The rhizosphere is a densely populated area in which the roots must compete with the invading root systems of neighboring plant species for space, water and mineral nutrients, and with soil borne microorganisms and insects feeding on an abundant source of organic material (Ryan and Delhaize, 2001). Thus, root-root, root-

microbe, and root-insect communications are likely continuous occurrences in this biologically active soil zone, but due to the underground nature of roots, these intriguing interactions have largely been over looked. Root-root and root-microbe communication can either be positive (symbiotic) to the plant, such as the association of epiphytes, mycorrhizal fungi and nitrogen-fixing bacteria with roots; or negative to the plant, including interactions with parasitic plants, pathogenic bacteria, fungi, and insects.

Rhizosphere contains lots of organic substrates which harbor a high count of organisms. It is a hot spot of microbial interactions as exudates released by plant roots are a main food source for microorganisms and a driving force of their population density and activities. Species richness is usually high in the rhizosphere and root free soil, but distinctly low in the rhizoplane around the root. A wide range of microorganisms are beneficial to the plant and include nitrogen fixing bacteria, phosphorous solubilizers, potasium mobilizers, mycorrhizal fungi and plant growth promoting bacteria and fungi. Generally, saprotrophs or biotrophs grow in rhizosphere in response to the carbon loss, but plant pathogens may also develop and infect a susceptible host, resulting in disease (Whipps, 2001). The number and diversity of deleterious and beneficial microorganisms are related to quantity and quality of the rhizodeposits and to the outcome of microbial interactions that occur in the rhizosphere (Somers *et al.*, 2004).

Because of the rapid consumption of nutrients, bacterial growth in the rhizosphere remains nutrient-limited where roots are seldom colonized for more than about 15% of their surface area. The rhizosphere microflora plays an important role in plant development and acclimation to environmental stresses. Since the rhizosphere microflora is extremely diverse, a dynamic interplay between the members of the microbial community occurs which is mediated by synergistic and antagonistic interactions within the limits of the nutrients available.

In the last few years, the number of PGPM that have been identified has seen a great increase, mainly because the role of the rhizosphere as an ecosystem has gained importance in the functioning of the biosphere.

2.3.1. Plant growth promoting microorganisms (PGPM)

PGPM are defined as root colonizing microorganisms that exert beneficial effects on plant growth and development. Root colonization comprises the ability of microorganism to establish on or in the plant root, to propagate, survive and disperse along the growing root in presence of the indigenous microflora.

Rhizosphere microoraganisms are considered as efficient microbial competitors in the root zone. Representatives of many different microbial genera have been introduced into soils, onto seeds, roots, tubers or other planting materials to improve crop growth. In addition to plant growth promotion, PGPM are also employed for controlling plant pathogens, enhancing efficiency of fertilizers and degrading xenobiotic compounds (rhizoremediation) (Kloepper *et al.*, 2004). Common PGPM include the strains in the genera *Acinetobacter, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Pseudomonas, Rhizobium* and *Serratia* (Lugtenberg & Kamilova, 2009).

Beneficial plant-microbe interactions in the rhizosphere can influence plant vigor and soil fertility. These beneficial effects of PGPM have direct or indirect performance on plants. Direct promotion of growth by PGPM including production of metabolites that enhances plant growth such as auxins, cytokinins, gibberellins and through the solubilization of phosphate minerals (Hadad *et al.*, 2010). Indirect growth promotion occurs via the removal of pathogens by the production of secondary metabolites such as hydrogen cyanide and siderophores.

PGPM are defined by three intrinsic characteristics (i) they must be able to colonize the root, (ii) they must survive and multiply in microhabitats associated with the root surface, in competition with other microbiota, at least for the time needed to express their plant promotion/protection activities and (iii) they must promote plant growth (Lugtenberg *et al.,* 2001). These are known to participate in many important ecosystem processes, such as the biological control of plant pathogens, nutrient cycling and seedling growth (Persello-Cartieaux *et al.,* 2003; Barea *et al.,* 2004; Zahir *et al.,* 2004). Some bacterial strains directly regulate plant physiology by mimicking synthesis of plant hormones, whereas others increase mineral and nitrogen availability in the soil as a way to augment growth. The isolates could exhibit more than two or three PGP traits, which may promote plant growth directly or indirectly or synergistically (Joseph *et al.,* 2007).

PGPM improve plant growth not only by increasing nutrient uptake and providing roots with phytohormones and solubilized iron but also by suppressing either major or minor pathogens of plant through an array of mechanisms (Cook and Rovera, 1976). Several studies have shown that PGPM not only benefit crops (Kurabachew and Wydra, 2013) but also improve the use efficiency of fertilizers and manures thus allowing reduced application rates (Dinesh *et al.,* 2013).

The variability in the performance of PGPM may be due to various environmental factors that may affect their growth and exert their effect on the plant. The environmental factors include climate, weather conditions, soil characteristics or the composition or activity of the indigenous microbial flora of the soil. To achieve the maximum growth promoting interaction between PGPR and nursery seedlings it is important to discover how the rhizobacteria exerting their effects on plant and whether the effects are altered by various environmental factors, including the presence of other microorganisms (Bent *et al.,* 2001). Diverse arrays of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter* and *Bacillus* have been

shown to promote plant growth and improve soil structure (El-Komy, 2005; Yasari and Patwardhan, 2007).

Species of *Pseudomonas* comprise a large portion of the total culturable bacterial population in the rhizosphere. Glick *et al.* (1995) reported that P solubilization, biological nitrogen fixation improvement of other plant nutrients uptake and phytohormone production like indole acetic acid (IAA) are some examples of mechanisms of PGPMs, that directy influence plant growth. Some strains of beneficial PGPM trigger plant mediated induced systemic resistance (ISR) response effective against a broad spectrum of plant pathogens (Saharan and Nehra, 2011).

Moreover, numerous actinomycetes are also one of the major components of rhizosphere microbial communities displaying marvelous plant growth beneficial traits (Merzaeva and Shirokikh, 2006). Among them, *Micromonospora* sp., *Streptomyces* spp., *Streptosporangium* sp. and *Thermobifida* sp., which have shown an enormous potential as biocontrol agents against different root fungal pathogens, are worthy of mention (Franco-Correa *et al.*, 2010).

Use of PGPM offers an attractive way to replace chemical fertilizer, pesticides, and supplements. Application of these results in a significant increase in plant height, root length, and dry matter production of shoot and root of plants. Utilization of PGPM in order to increase the productivity may be a viable alternative to organic fertilizers which also helps in reducing the pollution and preserving the environment in the spirit of an ecological agriculture (Stefan *et al.*, 2008).

2.3.2. Mechanisms of plant growth promotion

Growth promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the rhizosphere and other PGP activities (Arshad and Frankenberger, 1993; Glick, 1995).

The PGPM strains facilitate growth of plants either directly or indirectly. The direct mechanism of plant growth promotion involves the production of substances by bacteria and its transport to the developing plants or facilitates the uptake of nutrients from the recipient environment (Azcon, 1989). The direct growth promoting activity of PGPM includes (i) N_2 fixation (ii) solubilization of insoluble phosphorus (iii) sequestering of iron by production of siderophores (iv) production of phytohormones such as, auxins, cytokinins, gibberellins and (v) lowering of ethylene concentration (Liu *et al.*, 2007). On the contrary, the indirect mechanism of plant growth promotion by PGPM includes (i) antibiotic production (ii) depletion of iron from the rhizosphere (iii) synthesis of antifungal metabolites (iv) production of fungal cell wall lysing enzymes (v) competition for sites on roots and (vi) induced systemic resistance (Saravanakumar *et al.*, 2007; Cazorla *et al.*, 2007).

2.3.2.1. Production of plant hormones

Plant hormones are chemical messengers that affect a plant's ability to respond to its environment. Hormones are organic compounds that are effective at very low concentration; they are usually synthesized in one part of the plant and are transported to another location. They interact with specific target tissues to cause physiological responses, such as growth or fruit ripening. Each response is often the result of two or more hormones acting together. Because hormones stimulate or inhibit plant growth, many botanists also refer to them as plant growth regulators. Botanists recognize five major groups of hormones: auxins, gibberellins, ethylene, cytokinins, and abscisic acid.

2.3.2.1.1. Indole acetic acid (IAA) production

IAA is a member of the group of phytohormones and is generally considered the most important native auxin. Auxin biosynthesis is also widespread among soil and plant associated bacteria. IAA is considered to be the most important auxin that regulates plant growth and development. It is responsible for division, enlargement and differentiation of plant cells and tissues; it plays a major role in xylem and root formation (Davies, 1995). Moreover, its production is a determinant trait both for PGPM and plant pathogens (Patten and Glick, 2002).

Some microorganisms produce auxins in the presence of a suitable precursor such as L-tryptophan. Tien *et al.* (1979) showed that *Azospirillum* is able to produce auxins when exposed to tryptophan (Trp). The tryptophan increased the production of IAA in *Bacillus amyloliquefaciens* FZB42 (Idriss *et al.*, 2002). Plants inoculated with the rhizobia together with Ag+ ion and L-tryptophan gave the highest root dry weight and significantly increased the uptake of N, P and K compared to non-inoculated control plants (Etesami *et al.*, 2009). Karnwal (2009) tested Fluorescent pseudomonads isolates for their ability to produce indole acetic acid in pure culture in the absence and presence of L-tryptophan and found that for both strains, indole production increased with increases in tryptophan concentration.

The highest concentration of IAA was produced by bacterial strain *P. fluorescens* and *Kocuria varians* (Ahmad *et al.*, 2005). While working on chickpea they were found that all the isolates of *Bacillus, Pseudomonas* and *Azotobacter* produced IAA, whereas only 85.7% of *Rhizobium* was able to produce IAA (Joseph *et al.*, 2007). *Pseudomonas fluorescens* B16, a plant growth promoting rhizobacterium, produced Pyrroloquinoline Quinone, which is a plant growth promotion factor (Choi *et al.*, 2008). However, the ability of *Azotobacter* to produce plant growth promoting substances such as phytohormone and IAA was attributed more to yield improvement rather than to diazotrophic activity.

The role of IAA for better and profuse rooting in various plants is well documented. Diverse soil microorganisms including bacteria and filamentous fungi are capable of producing physiologically active quantities of auxins and which have pronounced effects on plant growth and development. Previous studies on biological nitrogen fixation by Chaiharn *et al.* (1928) revealed the role of IAA produced by rhizobacteria in increasing the absorption of nutrients by increasing the production of root hairs by the plant. Most root promoting bacteria synthesise IAA and it has been clearly demonstrated that they stimulated the formation of lateral and adventitious roots (Barbieri and Galli, 1993). Another report by Patten and Glick (1996) revealed that 80% of bacteria isolated from the rhizosphere could produce IAA. Similarly, higher level of IAA production by *Pseudomonas* and *Klebsiella* was reported by other research workers (Xie *et al.,* 1996). The other evidences include that the *P. putida* GR 12-2 cells that produced wild type levels of IAA stimulated the formation of many short adventitious roots on mung bean cuttings and in IAA over producing mutant stimulated the formation of even more adventitious roots than the wild type strain (Mayak *et al.,* 1997).

In the rhizosphere, production of ethylene results in inhibition of root elongation. In plants treated with rhizobacteria, the production of ethylene was inhibited by aminocyclo propane carboxylic acid deaminase which resulted in production of ammonia instead of ethylene. This resulted in rapid elongation of roots (Kloepper, 2003). Repeated application of *P. fluorescens* might have increased the auxin pool near to the root zone resulting in increased root and biomass production. Shanthi *et al.* (2003) reported that soil application of *P. fluorescens* resulted in higher plant height, number of leaves, root length and root weight compared to rhizome dip in banana.

2.3.2.1.2. Nitrogen fixation

Nitrogen (N) is the most vital nutrient for plant growth and productivity. Although, there is about 78% N_2 in the atmosphere, it is unavailable to the growing plants. Atmospheric N_2 is converted into plant-utilizable forms by biological N_2 fixation (BNF) which changes nitrogen to ammonia by nitrogen fixing microorganisms using a complex enzyme system known as nitrogenase (Kim and Rees, 1994). Plant growth promoting rhizobacteria that fix N_2 in non-leguminous plants are also called as diazotrophs capable of forming a non-obligate interaction with the host plants (Glick *et al.,* 1999). The use of bio-fertilizer and bioenhancer such as nitrogen fixing bacteria and beneficial microorganisms can reduce chemical fertilizer applications and consequently lower production cost.

Azotobacter is generally regarded as a free-living aerobic nitrogen fixer. *Azotobacter paspali,* which was first described, by Dobereiner and Day (1975) has been isolated from the rhizosphere of *Paspalum notatum*, a tetraploid subtropical grass and is highly host specific. Various crops in India have been inoculated with diazotrophs particularly *Azotobacter* and *Azospirillum* (Tilak and Saxena, 2001). Reports prove that application of *Azotobacter* and *Azospirillum* improves the yield of both annual and perennial grasses.

Members of the genus *Azospirillum* fix nitrogen under microaerophilic conditions and are frequently associated with root and rhizosphere of a large number of agriculturally important crops and cereals. Although they posses N_2 fixing capability (~1–10 kg N/ha), the increase in yield is mainly attributed to improved root development due to the production of growth promoting substances and consequently increased rates of water and mineral uptake (Dewan and Subha Rao, 1979; Okon and Kapulnik, 1986; Fallik *et al.,* 1994)

Azoarcus, which is an aerobic/microaerophilic nitrogen-fixing bacterium, was isolated from surface-sterilized tissues of Kallar grass (*Leptochloa fusca* L Kunth)

(Reinhold *et. al.,* 1986) and can infect roots of rice plants as well Kallar grass used as a pioneer plant in Pakistan on salt-affected low fertility soils as it is a salt-tolerant grass. The genus *Azoarcus* has been identified with two species, *A. indigens* and *A. communis* and three additional unnamed groups, which were distinct at species level. Nitrogen fixation by *Azoarcus* is extremely efficient.

Nitrogen fixation has been described in some species of *Paenibacillus,* such as *Paenibacillus polymyxa* (Grau and Wilson, 1962), *Paenibacillus* macerans (Witz *et al.,* 1967), *Paenibacillus azotofixans* (Seldin *et al.,* 1984), *Paenibacillus peoriae* (Elo *et. al.,* 2001), *Paenibacillus graminis, Paenibacillus odorifer* (Berge *et al.,*2002) and *Paenibacillus brasilensis* (Von der wied *et al.,* 2002). It has been reported that *Paenibacillus azotofixans* has the highest nitrogenase activity among these nitrogen fixers.

2.3.2.1.3. Phosphorus solubilisation

Phosphorus (P) is a major essential macronutrient for biological growth and development. Microorganisms offer a biological rescue system capable of solubilising the insoluble inorganic P of soil and make it available to the plants. The ability of some microorganisms to convert insoluble P to an accessible form, like orthophosphate, is an important trait in a PGPM for increasing plant yield (Rodriguez et *al.,* 2006). Among the heterogeneous and naturally abundant microbes inhabiting the rhizosphere, the phosphate solubilising microorganisms (PSM) including bacteria have provided an alternative biotechnological solution in sustainable agriculture to meet the P demands of plants. These organisms in addition to providing P to plants also facilitate plant growth by other mechanisms.

PSM include largely bacteria and fungi. The most efficient PSM belong to genera *Bacillus, Rhizobium* and *Pseudomonas* amongst bacteria, and *Aspergillus* and *Penicillium* amongst fungi. Within rhizobia, two species nodulating chickpea, *Mesorhizobium ciceri* and *Mesorhizobium mediterraneum*, are known as good

phosphate solubilizers (Rivas *et al.,* 2006). However, it is known that every aspect of the process of nodule formation is limited by the availability of P.

It is generally accepted that the mechanism of mineral phosphate solubilisation by PSM strains is associated with the release of low molecular weight organic acids (Goldstein, 1995; Kim *et al.,* 1997), which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, there by converting it into soluble forms. However, phosphorus solubilisation is a complex phenomenon which depends on many factors such as nutritional, physiological and growth conditions if the culture (Reyes *et al.,* 1999).

Identification and characterization of soil PSM for the effective plant growth promotion broadens the spectrum of phosphate solubilises available for field condition. Bacterial isolates *Pseudomonas* sp. and *Azospirillum* sp. from the rhizosphere soil and root cuttings of *Piper nigrum* L. exhibited high phosphate solubilising ability *in vitro* (Ramachandran *et al.,* 2007). The application of PSM and PGPM together reduced P application by 50% without any significant reduction of grain yield in corn Zea mays (Yazdani *et al.,* 2009).

2.4. MAJOR DISEASES OF BLACK PEPPER

The production of black pepper is hampered by diseases not only in India but also in other black pepper growing countries. Among these, foot rot and yellowing are the most important ones affecting black pepper production.

2.4.1 Yellowing of black pepper

Yellowing is a debilitating disease of black pepper. Foliar yellowing, defoliation and die back are the aerial symptoms of this disease. The exact cause of yellowing is unknown. It is considered as a complex disease caused by infestation of plant parasitic nematodes, soil borne pathogens and nutrient imbalance. However, some plants in the diseased garden affected by either foot rot or yellowing remain

healthy. This may be due to the inherent genetic ability of the plant or by the effect of rhizosphere microflora on their growth.

2.4.2. Foot rot disease of black pepper

Foot rot is caused by the soil-borne fungal pathogen *Phytophthora capsici.* Since the pathogen is soil borne, native antagonistic microorganisms play a major role in keeping the population of pathogen at low levels. On global scale, an annual crop loss of $4.5-7.5million has been reported due to foot rot alone (De-Waard, 1979). Crop loss due to foot rot in Kerala was estimated to range from 3.4-9.4% (Anadaraj *et al.,* 1989).

The disease is prevalent in Kerala, Karnataka, and Tamil Nadu states and recently in Assam (Sarkar *et al.*, 1985). It is a debilitating disease and affected plants survive for several years and death of plants occur gradually over a period of 4 years (Ravindran, 2000). The term quick wilt was vague to describe foot rot earlier because of quick death of black pepper vines due to the infection. It is the most destructive disease prevalent in all pepper growing tracts of India and takes a heavy toll of the crop in all countries were pepper is grown. The losses are so heavy that farmers get disheartened and abandon the crop. Even though the infection remains a perennial threat throughout the growth of the crop, it becomes predominant during the wet conditions of tropical monsoon (Sarma and Premkumar., 1988).

Phytophthora foot rot is more severe in the orchards if nematodes such as *Radopholus similis* and *Meloidogyne incognita* are present together with the causal fungus (Anandaraj *et al.*, 1996). Nematodes have also been reported as a major constraint to the black pepper production. A survey conducted by Eng (2001) in 43 black pepper farms revealed that root-knot nematodes (RKN) were present in all farms, suggesting that black pepper was one of the important hosts of RKN.

2.4.2.1. Phytophthora capsici

The scientific name *"Phytophthora"* (pronounced "fy-tof-thora" - derived from Greek "phyton" meaning "plant" and "phthora" meaning "destruction"). *P. capsici* is a soil borne fungus that belongs to the family Pythiaceae and cause serious root rot and foliar blight diseases on plants. It was first described as a pathogen of pepper in New Mexico (Leonian, 1922). The disease occurs mainly during the South-West monsoon period, (June to September) when weather conditions are favorable. High soil moisture, high relative humidity, shorter duration of sunshine hours, well distributed rainfall and low temperature is very conducive for the disease development (Sarma et *al.*, 2002)

P. capsici produces hyphae, which are without cross walls (coenocytic). It produces sporangiophores, sporangia and zoospores (asexual reproductive bodies) and antheridia, oogonia, and oospores (sexual reproductive bodies). It produces abundant sporangia on culture media at 20-25°C (Islam *et al.*, 2004).

Under favorable conditions of moisture and temperature, sporangia produce zoospores. In the moist soil, sporangia release zoospores into the soil. *P. capsici* also forms chlamydospores (thick walled cells), which survive in soil under unfavorable environmental conditions. However, not all isolates of *P. capsici* are capable of forming chlamydospores (Ristaino, 1990). It is a heterothallic oomycete requiring two different mating types (A1 and A2) for oospore production. Both of the mating types can produce antheridia (male reproductive structure) and oogonia (female reproductive structure). Oospores of *P. capsici* undergo a maturation phase during which nuclei fuses before oospores germinate.

P. capsici oospore germination was reported to increase with age (Satour and Butler, 1968). Lower rate of germination was reported for oospores produced in dark conditions but exposed to light for a week before harvesting from the culture medium, compared to the oospores under continuous dark conditions until harvested

from the plates. The best temperature for *P. capsici* oospore germination was reported to be 24°C (Hord and Ristaino, 1991).

2.4.2.2. Disease cycle and epidemiology of Phytophthora blight

P. capsici overwinters in soil as oospores and as mycelia in infected plant debris (Erwin and Ribeiro, 1996). Oopores can survive in the soil for more than three years, while survival of mycelia does not exceed four months. Under warm and wet conditions oospores in the soil germinate to form sporangia and sporangia produce zoospores. Spread of sporangia occurs by water and by aerial dispersion (Ristaino and Johnston, 1999). Plants may be infected by sporangia directly or by zoospores via direct contact or through splashing irrigation and rainfall (Granke *et al.*, 2009). Zoospores, released into saturated soil with water, swim and infect the plants and produce mycelia, sporangiophores, and sporangia on the infected parts.

The life cycle of *P. capsici* is repeated in every few days and disease spreads rapidly. It can grow at a temperature range of 7-37°C and sporangia and zoospores are abundantly produced at a temperature range of 27-32°C (Roberts *et al.*, 2000). Surviving mycelia on plant debris, in the soil, also cause infection in crops. Mycelia grow to form sporangia, which produce zoospores to infect plants.

2.4.2.3. Host Range

P. capsici has a wide host range that includes a minimum of 53 susceptible species in 24 different families. The families Cucurbitaceae and Solanaceae contain many horticulturally significant crops worldwide that are susceptible to *P. capsici* (Hausbeck and Lamour, 2004). Arecanut, coconut, cocoa, rubber, black pepper, betel vine and vanilla are the most important plantation crops that have been seriously affected by *Phytophthora* species.

2.4.2.5. Symptomatology

The detailed symptomatology of the disease was described by Sarma and Nambiar (1982). The *P. capsici*, a soil borne fungus, infects all parts of the black pepper vine, i.e., leaves, stem, inflorescence, collar and roots that led to leaf rot, stem rot, dropping of inflorescence, collar rot and root rot respectively. Among these infections, collar rot and root rot cause severe and sudden mortality of the vines.

Infection by *P. capsici* in nursery may result in either wilting of the cuttings or rotting of tender leaves. The former is caused mainly by inoculum of the fungus in the planting material and later through air borne sporangia. The disease is often carried to main field through the infected rooted cuttings and rooting medium. *P. capsici* infects all parts of the plant including the root, spike, leaves and shoot.

Leaf infection of black pepper vines appears as grey centers, surrounded by alternating dark and light brown zones with peripheral water soaked margins. This dual zonation occurred in alternate wet and dry weather but not in continuous wet condition. Wilting and rapid defoliation generally were the first symptom of the disease (Alconero *et al.*, 1972), the tender leaves were highly susceptible than mature ones and the lower surface of leaf was more susceptible than upper surface (Turner, 1969).

The infection first appears on tender shoot tips or leaves of runner shoots arising from the base of the black pepper vines spreading on the ground. (Sarma *et al.* 1988). Later the infection spreads and reaches to leaves at lower region of the bush. Gradually, the infection spreads to the upper regions of the vine including spike. Foliar infection is more serious in areca-pepper mixed cropping system because of the conducive macroclimate.

Spike infection at the distal end results in severe spike shedding by abscission. Black lesions are developed at distal end of the spike and infecting occasionally a few berries only.

Stem infection as dark brown lesions on the lateral branches result in wilting and shedding. The tender berries on the spikes borne on such infected branches become shriveled and the spikes are shed. Breaking of the nodal region of the stem is characteristic symptom.

The collar and root infection are fatal and the infected vines die within 10-20 days. Hence, the disease is locally called "quick wilt'. The collar rot infection occurs either at the collar or just above or below the soil level. Collar and root infection go unnoticed until foliar yellowing is recognized. The infection at initial stage starts as water soaked which is same as leaf infection. The lesions later turn brown to dark brown within two to three days and appear as slimy dark patch. Young leaves become flaccid followed by yellowing and defoliation. The affected portion is wet discolored slimy emitting foul smell. Vascular discolorations observed in many cases but not consistently (Nambiar and Sarma, 1977). During the advanced stage of infection the cortex get disintegrated and peeled off. The infection of the collar gradually progress downwards and spreads to the root system. This results in rotting of the root.

Root infection of the vines goes unnoticed without any visible aerial symptoms. The rootinfection starts on finer feeder roots, later spreads to the main roots and the collar. The black pepper vines remain healthy until large portions of the roots are damaged. In the advanced stages of the root rot, foliar yellowing of the vine, and shedding of leaves, spikes and lateral branches are noticed. The amount of defoliation due to root rot infection is equal to root damage. The root loss to regeneration determines the spread of the decline and death of the vine.

During the post monsoon season with depletion of soil moisture, the remaining root system is unable to support the vine, so the entire vine collapse with

wilting and drying of leaves. Foliar yellowing, flaccidity, defoliation, breaking of the stems at nodal regions and spike shedding are the characteristic aerial symptoms of root rot and collar rot infections (Muller, 1936 and Holliday and Mowat 1963).

2.4.2.6. Disease Management

Proper drainage in the field is a key factor for controlling *P. capsici* in the field (Ristaino and Johnston, 1999). A level, well drained, field with no low lying areas will help prevent focal points for the development of epidemics. A proper irrigation plan will also help reduce the incidence of disease in the field.

A study conducted by Filho et al. (1995) showed that avoiding excessive irrigation reduced the loss in yield due to *P. capsici* in a furrow field. Furrow irrigation should also be limited due to the easy spread of *P. capsici* in water from the point of origin down the field to non-infected plants.

Among the different chemical formulations tested, spraying and drenching with 1 per cent Bordeaux mixture before and after onset the of monsoon and pasting with 10 per cent Bordeaux paste to treat the stem collar were found effective for wilt of black pepper (Nambiar and Sarma, 1977). In the *in vitro* screening against *Phytophthora* capsici and *Phytophthora meadii* Mc Rae, Bordeaux mixture (1%), Blitox and Metalaxyl were found effective in inhibiting growth and sporangial formation (Sastry, 1982).

However, in a conducive environment, these fungicides have been proven inadequate in controlling this pathogen. Mefenoxam, a systemic phenylamide chemical used in many fungicides by growers, has become ineffective in controlling *P. capsici* due to a possible single gene mutation in the pathogen (Lamour and Hausbeck, 2003).

Nair and Sasikumaran (1991) reported that, among 5 fungicides tested to control the infection of *Piper nigrum* by *P. capsici*, Bordeaux mixture gave the best control followed by metalaxyl, copper oxychloride and captafol.

Veena and Sarma (2000) studied the uptake and persistence of potassium phosphonate recommended for the control of foot rot of black pepper. Potassium phosphonate at 500ppm, 1000ppm and 2000 ppm concentrations, both as aerial spray and soil drench, indicated that maximum reduction of foliar in infection (up to 86.4%) was noticed 4 days after treatment whereas root rot suppression (up to 70%) 8 days after treatment. They also reported prolonged protection beyond 30 days could be obtained with 2400 ppm to 4000 ppm concentration. There was no phytotoxicity on black pepper even at 4000 ppm.

Furthermore, the growing cost of chemicals, particularly in less affluent regions of the world and consumer demand for chemical free food has led to a search for substitutes for these products. There are also a number of fastidious diseases for which chemical solutions are few, ineffective, or nonexistent. Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture.

2.5. BIOLOGICAL CONTROL

As agricultural production intensified over the past few decades, producers became more and more dependent on agrochemicals as a relatively reliable method of crop protection helping with economic stability of their operations (Compant *et al.*, 2005).

However, increasing use of chemical inputs causes several negative effects, i.e., development of pathogen resistance to the applied agents and their nontarget environmental impacts (Werger *et al.*, 1995). There are also a number of fastidious diseases for which chemical solutions are few, ineffective, or nonexistent

(Gerhardson, 2002). Biological control is thus being considered as an alternative or a supplemental way of reducing the use of chemicals in agriculture (Welbaum, 2004).

Microorganisms as biocontrol agents have high potential to control plant pathogens and have no negative effect on the environment and other non target organisms. Biocontrol with plant growth promotion helps increasing the vegetative yield and thereby increasing crop yield. PGPM can suppress diseases through antagonism between bacteria and soil-borne pathogens, as well as by inducing a systemic resistance in the plant against both root and foliar pathogens. The induced resistance constitutes an increase in the level of basal resistance to several pathogens simultaneously, which is of benefit under natural conditions where multiple pathogens exist (Loon and Glick, 2004).

Various microorganisms which are predominantly studied and increasingly marketed as the biological control agents includes the genera *Bacillus*, *Streptomyces*, *Pseudomonas*, *Burkholderia* and *Agrobacterium*. They suppress plant disease through at least one mechanism; induction of systemic resistance, and production of siderophores or antibiotics.

Fluorescent pseudomonads and *Trichoderma* from rhizosphere of pepper were screened *in vitro* for their antagonistic potential against *P. capsici* using dual culture technique (IISR, 2000). Inhibition ranged from 26.9 to 37.6 per cent in *Trichoderma* spp. and 36.3 to 70 per cent in fluorescent pseudomonads. Fluorescent pseudomonad antagonistict o *P. capsici* were also good phosphate solubilizers, which is an additional advantage.

Trichoderma harzianum and *Trichoderma viride* have been used as biological control agents against *P. capsici* attacking black pepper, both in the main field and nursery (Anandaraj and Sharma, 1995). Only limited attempts have been made on the biological control of foot rot of black pepper using bacterial antagonists (Jubina and Girija, 1998). Studies have been conducted in other crops also. PGPMs including

two bacteria and one actinomycete isolated from bamboo rhizosphere increased the growth of Bengal gram plants (Niveditha *et al.*, 2008).

Despite inconsistency in field performance, biological control is considered as an alternative or a supplemental way of reducing root diseases in agro-ecosystem (Sharma and Johri, 2003).

2.5.1. Bacteria as biocontrol agents

An improved *in planta* assay involving the pathogen, host plant and bacterial antagonist was proposed as an effective selection method for antagonistic bacteria against *P. capsici* infection in black pepper (Dinu *et al.*, 2007).

Fluorescent pseudomonads are effective candidates for biological control of soil borne plant pathogens owing to their versatile nature, rhizosphere competence and multiple modes of action besides being endophytic in the plant system including black pepper (Kloepper *et al.*, 1981, Weller *et al.*, 1988, Diby *et al.*, 2001).

Jubina and Girija (1998) recorded high population of bacteria from rhizosphere soils of healthy plants in diseased pepper plantation of PRS, Panniyur and wild pepper plants in Silent valley. Of 194 isolates, 15 showed antagonism to *P. capsici in vitro*. B13 and B5 were effective in reducing the disease in pepper plants. B7 was found to have dual roles like disease suppression and growth promotion. In another study, Rubio *et al.* (2008) reported that *P. fluorescens* inhibited *Phytophthora infestans* to an extent of 74%. They also reported a clear zone of inhibition in the dual plate is suggestive of production of antagonistic metabolites by *P. fluorescens*.

Anith *et al.* (2003) screened 64 bacterial isolates against *P. capsici* and isolates PN032 and *Pseudomonas* sp. showed high inhibition of fungal growth in dual culture studies. It has been further tested in shoot assay and *Pseudomonas* sp. showed maximum lesion suppression, but PN-032 which represented high inhibition in dual culture and poor inhibition in shoot assay. Thus, the ability of the antagonists to

suppress the lesion in shoot assay could be correlated with their biological control potential. Narasimhan and Sivakumar (2012) reported that the *Bacillus* sp. from the chilli rhizosphere showed broad spectrum antagonism against *Alternaria* sp. (55%), *Colletotrichum gloeosporioides* (57%), *Phytophthora capsici* (62%), *Rhizoctonia solani* (42%), *Fusarium solani* (42%), *Fusarium oxysporum* (40%) and *Verticillium* sp. (36%).

2.5.2. Actinomycetes as biocontrol agents

The actinomycetes group is an enclosed species of gram positive bacteria broadly distributed in both terrestrial and aquatic ecosystems and well known for the production of many important bioactive metabolites (Omura, 1986). The diversity and activity of terrestrial *Streptomyces* species have been estimated on different ecosystems, such as tropical soils Gomes *et al.* (2000), beach and dune systems and saline farmlands (Sajid *et al.*, 2009). Biological control products based on actinomycetes have been developed to suppress the growth of pathogenic microorganisms in fields. Many species has been isolated from different ecosystems, and evaluated *in vitro* and *in vivo* against species of *Aspergillus*, *Fusarium*, *Trichoderma*, *Curvularia oryzae*, *Pyricularia orizae and Bipolaris oryzae* (Ningthoujam *et al.*, 2009).

Lim *et al.* (1991) demonstrated that the *S. padanus* strain TH-04 produced valinomycin, possessing antifungal activity against *P. capsici*. Similarly, Xiong *et al.* (2012) found that *Streptomyces padanus* strain JAU4234 possess antimicrobial activity against various Gram-positive and Gram-negative bacteria and fungi. They were also found that the strain produces an antifungal antibiotic fungichormin and an antitumor antibiotic like actinomycin X_2. Nguyen *et al.* (2012) reported that *Streptomyces griseus* H7602 from the rhizosphere soil recorded an inhibiton of 53.33% on *P. capsici* causing root rot of pepper (*Capsicum annuum*) and produced

active substance as well as several lytic enzymes including chitinase and β-1,3-glucanase *in vitro*.

Zahaed (2014) reported some *Streptomyces* sp have the potential to be used as biocontrol agents to control plant diseases such as, root rots on chilli by *P. capsici* or leaf blight on ornamentals caused by *Helminthosporium* or *Curvularia* species. In this sense, many species of *Streptomyces* are known to be potent producers of many antibiotics against soil-borne pathogens (Narayana and Vijayalakshmi, 2009; Nonoh *et al.*, 2010).

Furthermore, chitinase actinomycete producers have received attention due to its use as a biocontrol agent (Bressan, 2003). Also *Streptomyces* strains have been evaluated to reduce the incidence of seed pathogenic fungus *Aspergillus* sp. in maize Usha *et al.*. (2011) and to antagonize human pathogens such as *Candida albicans* and *A. niger*.

2.5.3. Fungi as biocontrol agents

Application of environment-friendly fungal biological agents is more suitable in view of sustainable agriculture and can be incorporated in integrated control programs. *Trichoderma* is one of the most commonly used genera of fungi in the biological control of plant diseases (Rosa and Herrera, 2009). Ninety percent of the antagonists used to control plant diseases belong to this genus (Benitez *et al.*, 2004). *Trichoderma* plays a crucial role in biocontrol management of cereals, pulse, oilseed, fruit, vegetable and spices crops diseases by producing various type of antagonistic compound which reduces the growth and infection caused by pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions, and enzyme secretion. (Meena, *et al.*, 2014)

The success of biocontrol largely depends on the ability of the antagonists to proliferate under conditions of the given environment. The establishment of an

antagonist in a soil or substrate and its subsequent proliferation may be an important factor in biological control of pathogens (Lewis and Papavizas, 1985). Hence, the application of native fungal strains in biocontrol may be advantageous or even desirable. The potential of *Trichoderma* spp. in foot rot management in black pepper has been clearly established.

Weindling (1932) reported the mycoparasitism of *Rhizoctonia solani* causing damping off disease by the hyphae of *Trichoderma*. He also described production of an antibiotic by the antagonist which was toxic to both *R. solani* and *Sclerotinia americana* and named it as gliotoxin. Nambiar and Sarma (1977) isolated *Trichoderma* spp. from roots of black pepper vines and noted the lysis of mycelium of *Phytophthora capsici* due to over growth of antagonist. Malajczuk (1983) described *Phytophthora* spp. as weak competitors and *Trichoderma* spp. as active parasites of *Phytophthora* spp. contributing to their breakdown and decay in soil.

Rajan *et al.* (2002) isolated a number of *Trichoderma* isolates and were screened both *in vitro* and *in vivo*. Isolates *T. virens*-12 and *T. harzianum*-26 were found more effective to control the foot rot disease and isolate *T. harizianum*-26 most adaptive to the rhizosphere of black pepper. Kavitha and Nelson (2013) reported antagonism of *Trichoderma viride* and *Trichoderma koningii* isolated from the rhizosphere soil of sunflower. In dual culture method, *Trichoderma viride* recorded maximum growth inhibition of 72.20% against *Fusarium oxysporum* whereas, *Trichoderma koningii* effectively inhibited *Pythium debarianum* (57.42%). Dwivedi et al. (2014) reported that *Trichoderma* sp. was most effective in inhibiting the growth of all the antagonistic soil fungi tested. *Trichoderma viride* inhibited the growth of *Fusarium solani* by 82% followed by *Penicillium rubrum* (74.8%), *P. italicum* (72.6%), *Aspergillus flavus* (64.2%) and *A. niger* (56.6%).

The use of *Trichoderma* product has both short term effects: immediate control of diseases and growth enhancement of crops as well as long term effects

which are demonstrated by the decrease in fungal pathogen inoculum in the field. Presently, *Trichoderma* based products are considered as relatively novel biological control agents which can help farmers to reduce plant diseases and increase plant growth.

2.5.4. Mechanism of biocontrol

The widely recognized mechanism of biocontrol mediated by PGPM is competition for an ecological niche/substrate, production of inhibitory allelochemicals and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens. Recently, research on mechanisms of biological control by PGPM revealed that several PGPM strains protect plants against pathogen infection through induction of systemic resistance, without provoking any symptoms themselves.

Noveriza and Quimio (2004) reported the antagonistic activities of mycoflora of black pepper rhizosphere from Philippines against foot rot pathogen. The mechanism behind antagonism included competition for nutrients, mycoparasitism and antibiosis.

2.5.4.1. Hydrogen cyanide (HCN) production

One group of microorganisms which acts as biocontrol agents of weeds include the deleterious rhizobacteria (DRB) that can colonize plant root surfaces and able to suppress plant growth. Cyanide is a dreaded chemical produced by them as it has toxic properties. Although cyanide acts as a general metabolic inhibitor, it is synthesized, excreted and metabolized by hundreds of organisms, including bacteria, algae, fungi, plants, and insects, as a mean to avoid predation or competition. A secondary metabolite produced commonly by rhizosphere pseudomonads is Hydrogen Cyanide (HCN), a gas known to negatively affect root metabolism and root growth and is a potential and environmentally compatible mechanism for biological

control of weeds (Heydari *et al.*, 2008). HCN is produced by many rhizobacteria and is postulated to play a role in biological control of pathogens.

The HCN production is found to be a common trait of *Pseudomonas* (88.89%) and *Bacillus* (50%) in the rhizospheric soil and plant root nodules and is a serious environmental pollutant and a biocontrol metabolite in *Pseudomonas* species. Production of HCN by certain strains of *Pseudomonas fluorescens* has been involved in suppression of soil borne pathogens (Voisard *et al.*, 1989). Flaishman *et al.* (1996) reported that overproduction of HCN may control many fungal diseases in wheat seedlings. Paul *et al.* (2005) studied the antagonistic action of 12 fluorescent pseudomonads against *P. capsici.* These 12 isolates were found to supress the pathogen through different modes namely, production of volatile and non volatile inhibitory metabolites including HCN and siderophore mediated mechanism. Kumar and Chandra (2008) also reported *in vitro* antagonism by HCN producing PGPR against sclerotia germination of *M. phaseolina.*

Ryall *et al.* (2009) reported production of secondary metabolite HCN by an entomopathogenic bacterium *Pseudomonas entomophila* and it was the main implication for biocontrol properties and pathogenecity exerted by other bacteria. Vanitha and Ramjegathesh (2014) screened the *P. fluorescens* strains against *Macrophomina phaseolina* (Tassi) Goid, the causal organism of coleus root rot. The results revealed that strain Pf1 recorded maximum inhibition of mycelial growth. They also found out the mechanisms behind the antagonism were HCN, siderophore and antibiotic production.

The entomopathogenic bacterium *Pseudomonas entomophila* produces HCN which is a secondary metabolite and is implicated in biocontrol properties and pathogenicity exerted by other bacteria (Ryall *et al.*, 2009). The *Pseudomonas fragi* CS11RH1 (MTCC 8984), a psychrotolerant bacterium produces hydrogen cyanide (HCN) and the seed bacterization with the isolate significantly increases the percent

germination, rate of germination, plant biomass and nutrient uptake of wheat seedlings (Selvakumar, 2009)

2.5.4.2. Ammonia production

Production of ammonia has been reported to be a mechanism of antagonism by several workers. Martin (1982) reported that the accumulation of ammonia in soil may increase in pH creating alkaline condition of soil at pH 9-9.5. This condition suppresses the growth of certain fungi and nitrobacteria due to its potent inhibition effect. He also reported that this will upset the microbial community and inhibit germination of spores of many fungi.

Trivedi *et al.* (2008) isolated *Pseudomonas corrugata*, a soil bacterium originally from a temperate site of Indian Himalayan Region (IHR) and examined for its antagonistic activities against two phytopathogenic fungi, *Alternaria alternata* and *Fusarium oxysporum*. They also observed production of ammonia by the isolate which could be responsible for its antagonistic action.

2.5.4.3. Siderophore production

Iron, one of the most essential microelements for virtually all living cells, is usually abundant in the environment, particularly in soils and natural aquifers. Siderophores (Greek: "iron carrier") are small, high-affinity iron chelating compounds secreted by microorganisms such as bacteria, fungi and grasses. (Neilands, 1980). Microbes release siderophores to scavenge iron from these mineral phases by formation of soluble Fe^{3+} complexes that can be taken up by active transport mechanisms. Siderophores are also important for some pathogenic bacteria for their acquisition of iron. Siderophores are amongst the strongest binders to Fe3+ known, with enterobactin being one of the strongest of these. Distribution of siderophore producing isolates according to amplified ribosomal DNA restriction analysis (ARDRA) groups, reveals that most of the isolates belong to Gram- negative

bacteria corresponding to the *Pseudomonas* and *Enterobacter* genera, and *Bacillus* and *Rhodococcus* genera are the Gram-positive bacteria found to produce siderophores (Tian *et al.*, 2009).

Hien *et al,* (1990) reported that siderophore production is involved in the antagonistic effect of *Pseudomonas aeruginosa* strain 7NSK2 against *Pythium* causing post emergence damping off. Tian *et al.* (2009) studied the distribution of siderophore producing isolates according to amplified ribosomal DNA restriction analysis (ARDRA) groups and revealed that most of the isolates belonged to the Gram- negative bacteria *Pseudomonas* and *Enterobacter* genera, and Gram-positive bacteria included *Bacillus* and *Rhodococcus* genera.

Production of siderophores by plant growth PGPM are detected via the chrome azurol S assay, a general test for siderophores, which is independent of siderophore structure. Siderophores are usually classified by the ligands used to chelate the ferric iron. The major groups of siderophores include the catecholates (phenolates), hydroxamates and carboxylates (e.g. derivatives of citric acid).

However, its bioavailability is relatively low, which is connected with a dramatically decreased solubility of ferric species under physiological pH values owing to their complete hydrolysis. This has resulted in the development of special biologically regulated mechanisms of Fe(III) solubilization, e. g., involving specific natural low-molecular-weight chelating agents (siderophores) which transport iron(III) to the cell surface in the form of a complex, with further Fe(III) release from the latter in the course of its reductive assimilation (Kamnev *et al.*, 2000).

The fluorescent pseudomonads are characterized by yellow green pigments that fluoresce under UV irradiation and function as siderophores termed pyoverdins, and pseudobactins (Meyer and Abdallah, 1978).

2.5.4.4. Mycoparasitism by fungal antagonists

Mycoparasitism is yet another mode of antagonism by the fungal antagonist in controlling the growth of pathogen. The complex process of mycoparasitism consists of several events, including recognition of the host, attack and subsequent penetration and killing.

Pugeg and Ian (2006) reported the mycoparasitic action of *Trichoderma* strains against *Phytophthora cinnamoni*. Several workers reported that during this process *Trichoderma* secretes CWDEs (cell wall degrading enzymes) that hydrolyze the cell wall of the host fungus, subsequently releasing oligomers from the pathogen cell wall (Kubicek *et al.*, 2001; Howell 2003; Woo *et al.*, 2006). Similarly, Zegeye *et al.* (2011) examined the hyphal interactions between *T. viride* and the *P. infestans* and revealed that *T. viride* hyphae coiled around and grew along the hyphae of *P. infestans*.

2.5.4.5. Induced Systemic Resistance (ISR)

Elicitation of ISR by plant-associated bacteria was initially demonstrated using *Pseudomonas* spp. and other gram-negative bacteria. Several reviews have summarized various aspects of the large volume of literature on *Pseudomonas* spp. as elicitors of ISR. Published results are summarized showing that specific strains of the species *B. amyloliquefaciens, B. subtilis, B. pasteurii, B. cereus, B. pumilus, B. mycoides*, and *B. sphaericus* elicit significant reductions in the incidence or severity of various diseases on a diversity of hosts.

ISR of plants against pathogens is a widespread phenomenon that has been intensively investigated with respect to the underlying signalling pathways as well as to its potential use in plant protection. Elicited by a local infection, plants respond with a salicylic dependent signalling cascade that leads to the systemic expression of a broad spectrum and long lasting disease resistance that is efficient against fungi, bacteria and viruses. Salicylic acid (SA) has an important role in the signaling pathway leading to ISR. After infection, endogenous levels of SA increase locally

and systemically, and SA levels increase in the phloem before ISR occurs. SA is synthesized in response to infection both locally and systemically; *de novo* production of SA in non-infected plant parts might therefore contribute to systemic expression of ISR (Heiland and Bostock, 2002). Compared to pathogens inducing SAR, non-pathogenic rhizobacteria inducing ISR trigger a different signal transduction pathway not dependent on the accumulation of the SA and activation of pathogensis-related (PR) genes but dependent on precipitation of ethylene and jasmonic acid.

The plant growth promoting *Pseudomonas* strains, which induced resistance systematically in watermelon to gummy stem rot, are investigated on their induced systemic resistance (ISR) related characteristics by Lee *et al.* (2001). Their work supports the concept that PGPM can protect plants against the pathogens by inducing defense mechanisms by iron-binding siderophore, HCN and other associates. The plant growth promoting rhizobacteria induced systemic protection against tomato late blight (Yan *et al.*, 2002). Under *in vitro* conditions *P. fluorescens* (ENPF1) and *P. chlororaphis* isolate (BCA) promotes plant growth and induce systemic resistance against stem blight pathogen *Corynespora cassiicola* in *P. amarus* (Msthiyazhagan *et al.*, 2004). The involvement of ISR is typically studied in systems in which the *Pseudomonas* bacteria and the pathogen are inoculated and remain spatially separated on the plant, e.g. the bacteria on the root and the pathogen on the leaf, or by use of split root systems. Since no direct interactions are possible between the two populations, suppression of disease development has to be plant mediated (Bakker *et al.*, 1987).

Materials and methods

3. MATERIALS AND METHODS

The study entitled 'Functional diversity of beneficial microorganisms from the rhizosphere of black pepper in Wayanad' comprising both laboratory and *in planta* experiments were carried out during the period 2012-2014 at department of Agricultural Microbiology, College of Horticulture, Vellanikkara. The materials used and the methodologies adopted in this study are presented below under the following sub headings.

3.1. MATERIALS

3.1.1. Chemicals, glasswares and plastic wares

The chemicals used for the study were supplied by agencies like Merck, SRL and HIMEDIA. Molecular biology reagents and buffers were purchased from Bangalore Genei Ltd. and Sigma-Aldrich India Ltd. All plastic wares were obtained from Tarson India Ltd.

3.1.2. Equipment items

The equipment items available at the Department of Agricultural Microbiology were used for the study. Sterilization of culture media was carried out in autoclave Equitron-7440 SLEFA (Eutech Instruments India). Incubation of the cultures was carried out in incubator-shaker (Merck-Genei-OS_2, Merck India Ltd.). Centrifugation was carried out in centrifuge (Eppendorf-5804R, Eppendorf, Germany). A compound binocular microscope (Leica-DM500) was used for viewing the morphology of microorganisms and for photomicrography. pH of culture media was checked by using pH meter (Cyberscan-Eutech, Eutech Instruments, India). DNA amplification was carried out in Eppendorf Mastercycler (Eppendorf, Germany). UV transilluminator (UVP-Benchtop Transilluminator, USA) was used to

visualize the gel. Microbial cultures were stored in ultra-low temperature deep freezer (Haier DW-86L90, Haier International Co. Ltd., China).

3.2. METHODOLOGY

3.2.1. Collection of rhizosphere soil samples from Wayanad district

Four healthy pepper gardens and four gardens each affected by foot rot and yellowing were identified in Wayanad district. Approximately 100g soil was collected from the rhizosphere of five healthy pepper vines from each garden in polythene bag and brought to laboratory. Global Positioning System (GPS) reading of the location was also recorded. Soil samples collected from each garden were thoroughly mixed and composite sample of 100g was drawn. These samples were then air dried and stored under refrigerated conditions for further enumeration and isolation of microorganisms.

3.2.2. Isolation of rhizosphere microflora

Enumeration of microorganisms in rhizosphere soil samples was carried out by serial dilution and plate count method (Johnson and Curl, 1972).

In order to standardize the dilutions to be used in serial dilution plate count method for enumeration of different functional groups of microorganisms, an experiment was conducted with 15 different media viz. nutrient agar, soil extract agar, luria agar, King's B agar, King's A agar, potato dextrose agar, Jensens's agar, Ashby's N free agar, Pikovskaya's agar, Kustur's agar, Kenknight and Munaier's agar and Martin's rose Bengal agar and oligotrophic media. Oligotrophic nutrient agar, potato dextrose agar and Kenknight and Munier's agar containing 50% concentration of original medium was used. The compositions of these media are given in Appendix I. Ten gram of soil sample was transferred aseptically into 250 ml conical flask containing 90 ml of sterilized distilled water and the contents were mixed thoroughly by shaking for five minutes. One ml of aliquot was then drawn and

transferred to 9 ml water blank containing distilled water. The suspension was shaken for one minute before further dilution. Dilutions of up to 10^{-6} were prepared. For bacteria, dilutions of 10^{-4}, 10^{-5} and 10^{-6}; for actinomycetes 10^{-3}, 10^{-4} and 10^{-5}; for fungi 10^{-3}, 10^{-4} and 10^{-5}; for N_2-fixers 10^{-2}, 10^{-3} and 10^{-4}; phosphate solubilizers 10^{-4}, 10^{-5} and 10^{-6}; for *Bacillus* 10^{-4}, 10^{-5} and 10^{-6} were used for plating. One ml of suspension from respective dilutions was transferred aseptically into Petri dishes. Twenty ml of molten and cooled agar media was then poured into Petri dishes. The plates were rotated manually for uniform distribution of the suspension in medium and were allowed to solidify. Number of colonies on the respective dilution was calculated and expressed as colony forming units per gram of soil (cfu/g). Depending on the number of colonies on each media, the following dilutions were selected for further experiments. List of media and dilutions taken were given in Table 1.

3.2.3. Purification and maintenance of isolates

A total of 207 predominant rhizosphere isolates were purified and maintained as per standard procedures, as outlined below.

Bacteria - The bacterial colonies developed in the dilution plates were streaked on their respective agar media and single colonies were obtained. Pure cultures obtained were maintained as agar slants and stabs at 4^0C in refrigerator and as glycerol stock (broth culture containing 40% glycerol) at -80^0C in deep freezer.

Actinomycetes - The single colonies of actinomycetes obtained were transferred to agar slants, stab and glycerol stock.

Fungi - The fungal colonies developed on dilution plates were transferred to potato dextrose agar (PDA). Pure cultures of fungi thus obtained were maintained in PDA slants.

Table 1. Media and dilutions used for serial dilution plate count for isolation of microorganisms

Sl. No.	Media	Target organism	Dilutions used
1	Nutrient agar	Bacteria	10^{-4} to 10^{-6}
2	Soil extract agar	Total bacteria	10^{-4} to 10^{-6}
3	Luria agar	*Bacillus*	10^{-4} to 10^{-6}
4	King's B agar	*Pseudomonas fluorescens*	10^{-4} to 10^{-6}
5	King's A agar	*Pseudomonas fluorescens*	10^{-4} to 10^{-6}
6	Potato dextrose agar	Fungi	10^{-3} to 10^{-5}
7	Jensen's agar	Nitrogen fixers	10^{-2} to 10^{-4}
8	Ashby's N-free agar	Nitrogen fixers	10^{-2} to 10^{-4}
9	Pikovskaya's agar	Phosphate solubilisers	10^{-4} to 10^{-6}
10	Kustur's agar	Actinomycetes	10^{-3} to 10^{-5}
11	Kenknight & Munaier's agar	Actinomycetes	10^{-3} to 10^{-5}
12	Martin's rose Bengal agar	Fungi	10^{-3} to 10^{-5}
13	*Oligotrophic nutrient agar	Bacteria	10^{-4} to 10^{-6}
14	Oligotrophic potato dextrose agar	Fungi	10^{-3} to 10^{-5}
15	Oligotrophic Kenknight agar	Actinomycetes	10^{-3} to 10^{-5}

*50% concentration of original medium

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

Isolates obtained were screened for various PGP activities like indole acetic acid (IAA) production, phosphate solubilization and nitrogen fixation under *in vitro* conditions. The composition of various reagents used for the study are given in Appendix II.

3.2.4.1. Screening for IAA production

IAA is one of the most physiologically active auxins produced by several microorganisms including PGPM. Hence all the isolates of bacteria, fungi and actinomycetes obtained in the study were screened for IAA production *in vitro*. Ten isolates identified as potential IAA producers in the preliminary screening were then used for further quantification of the production of IAA *in vitro*.

3.2.4.1.1. Screening of bacterial isolates and actinomycetes for IAA production

All the bacterial and actinomycete isolates were screened for the production of plant growth hormone IAA following Brick *et al.* (1988). Luria Bertani (LB) agar supplemented with 0.06 per cent sodium dodecyl sulphate (SDS) and one percent glycol was prepared and plated. The surface area of the agar medium was divided into squares of 2 cm x 2 cm by marking on the bottom of each plate. The overnight culture of each isolate grown on Luria agar was spotted in each square. The spotted plates were over laid immediately with sterile disc of Whatman No. 1 filter paper. Plates were incubated until the colonies reached the size of 0.5 to 2 mm in diameter. After the incubation period, the filter paper discs were removed from the plates and treated with Salkowski reagent (2 % of 0.5 M $FeCl_3$ in 35 % perchloric acid) by soaking in a Petri dish containing the reagent. The reaction was allowed to proceed until adequate colour developed. Bacteria producing IAA were identified by the formation of characteristic red halo around the colony on filter paper.

3.2.4.1.2. Screening of fungal isolates for IAA production

Czapek dox broth containing 0.5% tryptophan was prepared, sterilized, cooled and inoculated with the fungal cultures which were incubated for a period of 15 days at 28°C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (5 ml) was then tested with Salkowski's reagent and development of pink colour indicated the production of IAA.

3.2.4.2. Quantitative estimation of IAA production

In vitro auxin production by ten selected isolates of bacteria and fungi from preliminary screening experiment was estimated as IAA equivalent in the presence of L-tryptophan by using the protocol described by Khalid *et al.* (2004). For this purpose, 10 ml of nutrient broth was taken in 100 ml Erlenmeyer flasks, autoclaved and cooled. L-tryptophan was filter sterilized by passing through 0.2 µm membrane filter and adjusted at a concentration of 1.0 µg ml^{-1} to the liquid medium. One ml of 3 days old bacterial broth adjusted to a population of 10^7-10^8 cfu/ ml was inoculated in the medium. The flasks were plugged tightly and incubated for 10 days. Non-inoculated broth was maintained as control.

After incubation, the contents were filtered through Whatman No. 2 filter paper. Auxin compounds (IAA equivalent) were determined by spectrophotometer, using Salkowski's reagent. For measuring IAA equivalents, 2.0 ml filtrate was mixed with 2 drops of orthophosphoric acid and 4.0 ml of Salkowski's reagent (0.5 M $FeCl_3$ + 98.0 ml of 35% $HClO_4$). The contents in the test tubes were allowed to stand for 30 minutes for colour development. Colour was also developed with standard solutions of IAA with concentration ranging from 0, 20, 40, 60, 80 and 100 µg ml^{-1}. The intensity of colour was measured at 530 nm in a spectrophotometer. Standard curve was used for calculating the amount of auxin produced by the isolates.

3.2.4.2. Screening for phosphate solubilizing activity

All the isolates obtained were screened for phosphate solubilization on Pikovskaya's agar. Twenty microlitres of 24 h old bacterial isolates, 5 days old actinomycetes and fungal isolates were spotted on Pikovskaya's agar plate. Inoculated plates were then sealed properly and incubated for 7 days at 28±2^{0}C. The halo-zone and colony diameter were measured after 7 days of incubation. The ability to solubilize insoluble P was expressed as per cent solubilization efficiency (SE) (Nguyen *et al.*, 1992).

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

Where,

SD = Solubilization diameter (mm)

CD = Colony diameter (mm)

3.2.4.3. Quantitative estimation of phosphate solubilization

Ten microbial isolates positive for P-solubilization on Pikovskaya's agar in the preliminary screening were subjected to quantification of inorganic phosphorus. Flasks containing 50 ml Pikovskaya's broth were inoculated with 500µl of overnight grown culture of each isolate and incubated for 10 days at 28±2^{0}C. The amount of inorganic P released in broth was estimated after 14 days of incubation along with the uninoculated control by using phospho-molybdic blue colour method (Olsen *et al.*, 1954). The procedure is as follows.

Pikovskaya's broth cultures were centrifuged at 10,000 rpm for 10 min 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 was added followed by 0.4 ml of ANSA reagent. The

contents were mixed for 10 min for colour development. Intensity of blue colour was read in a spectrophotometer at 660 nm. The amount of available 'P' present in the broth was calculated using standard graph of different known concentrations of P using KH_2PO_4.

The pH of the supernatant was also recorded after 14 days of incubation so as to assess the lowering of pH from the initial value of 7.2.

3.2.4.4. Screening of bacterial isolates for nitrogen fixation in N-free medium

All the bacterial isolates were streaked on N-free Jensen's agar. The inoculated plates were then incubated at 28± 2^0C for three days. Isolates which were able to grow on N-free media were selected as nitrogen fixers. Based on the growth in the media nitrogen fixers were rated as excellent (++++), good (+++), moderate (++) and poor (+).

3.2.4.5.. Estimation of nitrogen fixed by micro-Kjeldahl method

Nitrogen fixation by five excellent nitrogen fixers obtained after screening was studied by micro-Kjeldahl method of Jackson (1973) and Bremner (1960). A loopful of 48 h old culture was inoculated in 5 ml of Jensen's broth contained in glass tubes and incubated for 48 h. One ml of this culture was inoculated in 50 ml of respective medium 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 containing K_2SO_4 and catalyst mixture ($CuSO_4$: 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. 10ml of 40% NaOH was added and condensed NH_3 was collected in 10ml boric

acid-indicator mixture (4% boric acid solution was prepared in hot water). 4ml of mixed indicator solution (0.2% bromocresol green + 0.2% methyl red in alcohol in 5:1 ratio) was added to 1000ml of 4% boric acid solution. The colour changed from reddish pink to bluish green as the NH_3 entered. After this process was over, it was titrated against 0.01N HCl till the solution turned black to reddish pink. A blank was also used for titration. Total nitrogen content of the cultures were 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 C source

3.2.5. Evaluation of selected PGPM for growth promotion in black pepper cuttings

The most promising and efficient PGPM including two nitrogen fixers, two phosphate solubilisers and two IAA producers were evaluated for their efficacy in enhancing growth of pepper cuttings in nursery. The experiment was conducted during July 2013 to December 2014 at Department of Agricultural Microbiology, College of Horticulture, Vellanikkara.

The treatment details of experiment were as follows

Design : CRD

Replications : 3*

Treatments : 8

Variety : Panniyur 1

Treatments:

T_1 : Selected nitrogen fixer-1 (YPTN-3; *Paenibacillus* sp.)

T_2 : Selected nitrogen fixer-2 (HVKN-6; *Paenibacillus* sp.)

T_3 : Selected P solubiliser-1 (HPLPSB-3; *Acinetobacter grimontii*)

T_4 : Selected P solubiliser- 2 (HPLF-5; *Gongoronella butleri*)

T_5 : Selected IAA producer-1 (HPLBC-6; *Providencia* sp.)

T_6 : Selected IAA producer-2 (HABC-3; *Providencia* sp.)

T_7 : *Pseudomonas fluorescens*-P1 (KAU reference culture)

T_8 : Absolute control

* Each replication had four poly bags, each with four cuttings.

3.2.5.1. Preparation of potting mixture and planting

The potting mixture was prepared with sand: soil: cow dung in the ratio of 1:1:1. Potting mixture was sterilized by adding 100ml of 2 % formaldehyde and covering with polythene film for 7 days so that the vapours were retained in potting mixture. This mixture was then raked thoroughly and left open for 15 days before planting. This sterile potting mixture was filled in polythene bags of size 35 x 17 cm

and 150 gauge thickness. Disease free two node cuttings of black pepper procured from farmers field were used for planting. Pepper cuttings were treated with PGPM inoculum and planted at with four cuttings per bag.

3.2.5.2. Preparation and application of PGPM

3.2.5.2.1. Preparation and application of bacterial inoculum

Inocula of six bacterial isolates were prepared by collecting individual colonies from the agar plates and then inoculating in 200 ml of respective broth media at 28 ± 2^0C for 48 h. Inoculum was then uniformly diluted to 10^6 cfu ml^{-1}. Basal cut ends of the runner shoots were dipped in the bacterial suspension for 30min and then planted. Soil was inoculated with bacterial suspension @ 30ml per vine at the time of planting and 45 days after planting.

3.2.5.2.2. Preparation and application of spore suspension of fungal inoculums

Six mm discs of fungal isolates were inoculated in 200 ml potato dextrose broth and incubated at 28 ± 2^0C for 7 days. After incubation, the inoculum was thoroughly mixed and uniformly diluted to get spore suspension of 10^6 cfu ml^{-1}. Basal portion of the cuttings were then dipped in the spore suspension for 30 min and then planted. Soil application at the rate of 30 ml per vine was also given at the time of planting and 45 days after planting.

3.2.5.3. Biometric observations

Observations on sprouting, length of vine and number of leaves were recorded at monthly intervals up to 6 months after planting. Root growth parameters were recorded at 6 months after planting. For this, destructive sampling was adopted. The polybags were torn and soil carefully removed without damaging the roots. Number, length, fresh weight and dry weight of roots were recorded.

3.2.5.3.1. Sprouting percentage

The number of cuttings sprouted in each treatment was counted at one month, two months and three months after planting to calculate the per cent sprouting.

$$\text{Per cent sprouting} = \frac{\text{Total number of cuttings sprouted}}{\text{Total number of cuttings planted}} \times 100$$

3.2.5.3.2. Length of vine

The distance from the base of the cutting at the soil level to the tip of the vine was taken as length of vine at 2 months, 4 months and 6 months after planting and expressed in centimeters.

3.2.5.3.3. Number of leaves

Total number of leaves on each cutting were counted at 2 months, 4 months and 6 months after planting and mean number of leaves was obtained.

3.2.5.3.4. Number of roots

After 4 months and 6 months of planting the cuttings were removed from polybags and number of roots were counted and the mean values were obtained.

3.2.5.3.5. Length of roots

After 4 months and 6 months of planting the cuttings were removed from polybags and length of roots were recorded in centimeters.

3.2.5.3.6. Fresh weight of roots

Roots were separated from the uprooted plants at 4 and 6 months after planting and fresh weight recorded in grams per plant.

3.2.5.3.6. Dry weight of roots

Root samples were oven dried at 65^0C for 72 h till a constant weight was achieved. The dry weight of roots of different treatments were recorded in grams per plant.

3.2.5. *In vitro* screening for antagonistic activity on *Phytophthora capsici*

3.2.5.1. Isolation and identification of pathogen

The standard tissue isolation technique (Tuite, 1969) with slight modification was followed for isolation of pathogen from black pepper leaves showing typical symptoms of foot rot disease. The samples were collected from pepper gardens of CoH, Vellanikkara. The infected tissues were cut into small pieces and surface sterilized with one per cent sodium hypochlorite for thirty seconds under aseptic conditions. These bits were washed in three changes of sterile water and transferred to Petri dishes containing 20 ml of molten and cooled carrot agar (CA) amended with streptomycin at a concentration of 20 µg per ml for one day to avoid bacterial contamination. As the fungal pathogen *Phytophthora capsici* is also sensitive to streptomycin, bits were transferred after one day to CA media devoid of the antibiotic and incubated at 20-25°C for five days.

3.2.5.2. Test for pathogenicity

The pathogenicity of isolate obtained was proved by following Koch's postulates. Mycelial disc of pathogen from seven day old culture grown on carrot agar was inoculated on the lower surface of the detached leaves of pepper variety, Panniyur-1 after giving pinpricks. Humidity was provided by placing moist cotton over it. The leaves were placed in polythene bags and incubated. Observations were recorded on the development of black water-soaked lesions. The pathogen was re-isolated from the artificially inoculated leaves for studying its morphological and

cultural characters. Pathogen was then maintained in slants and sub-cultured for further studies.

3.2.5.3. Screening of bacterial isolates against P. capsici

Selected bacterial antagonists were evaluated for antagonistic activity against *P. capsici* by dual culture technique (Utkhde, 1984). 8 mm disc of *P. capsici* was placed on PDA contained in Petri plate, leaving 2 cm from the edge and incubated at 28± 2^0C for 48 hours. After incubation bacterial antagonists were inoculated exactly at opposite side of the same plate by leaving 3-4 cm from the pathogen. Three replicates were maintained for each treatment. Plates with pathogen alone served as control.

After seven days of incubation, when the growth of pathogen in control plate reached maximum (82 mm diameter), the radial growth of the pathogen was measured. Per cent inhibition over control was worked out according to the equation given by Vincent (1927).

$$I = \frac{C - T}{C} \times 100$$

Where,

C: Growth of pathogen in control (mm)

T: Growth of the pathogen in dual culture (mm)

3.2.5.4. Screening of actinomycetes against P. capsici

The method for testing *in vitro* antagonism of 32 actinomycetes against *P. capsici* was carried out using gross streak assay method (Ahamed and Ahamed, 1963). For this, the pathogen was inoculated 3.5 cm apart from the actinomycetes two days after the inoculation of the actinomycetes and incubated for 7 days.

Observations were recorded and per cent inhibition was calculated as described as in section 3.6.3.

3.2.5.5. Screening of fungal antagonists against P. capsici

Fungal antagonists were tested for their antagonistic activity by the method given by Skidmore and Dickinson (1976). Dual culture plate method was adopted, after giving due consideration for the growth rate of both pathogen and potential antagonists. Mycelial disc (8 mm) of the pathogen from four day old culture grown on PDA was inoculated aseptically on one side of a Petri dish and incubated at 28 ± 2^0C for 48 hours. After this, 5 mm mycelial disc of the fungal isolate was placed in the same PDA plate, 3.5 cm away from the pathogen disc and incubated. Three replications were made for each isolate. Petri plate inoculated with mycelial disc of pathogen alone served as control. The growth measurements were taken at regular intervals after 24 hours of inoculation of the antagonists upto 7 days. The per cent inhibition of mycelial growth of the pathogen (I) was calculated using the formula suggested in section 3.2.5.3.

3.2.6. Mechanism of antagonism

3.2.6.1. Effect of volatile metabolites of the antagonists against P. capsici

Selected antagonists after screening were tested for the mechanism of antagonism.

3.2.6.1.1. Hydrogen cyanide (HCN) production

HCN production by antagonists was tested following the method of Bakker and Schipper (1987). The antagonistic bacteria were streaked on King's B agar media amended with glycine at 4.4g l^{-1} whereas fungal antagonists were spot inoculated. Sterile filter paper (Whatman No. 1) saturated with picric acid solution (2.5 g of picric acid; 12.5 g of Na_2CO_3, 1000 ml of distilled water) was placed in the upper lid

of the Petri plate. The dishes were sealed with parafilm and incubated at 28^0C for 5 days. A change of colour of the filter paper from yellow to light brown, brown or reddish-brown was recorded as weak (+), moderate (++) or high (+++) reaction respectively.

3.2.6.1.2. Ammonia (NH_3) production

Bacteria, fungi and actinomycetes obtained were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 4 per cent peptone water in each tube and incubated at 28 ± 2^0C. Bacterial isolates were incubated for 2 days and fungi as well as actinomycetes for 4 days. After incubation, Nessler's reagent (0.5 ml) was added in each tube. Development of yellow to brown colour was a positive test for ammonia production (Cappuccino and Sherman, 1992). Based on the intensity of colour produced, the reaction was rated as follows:

Yellow: weak (+)

Orange: moderate (++)

Brown: high (+++).

3.2.6.2. Effect of non-volatile metabolites

Selected isolates of bacteria, fungi and actinomycetes from the preliminary screening for antagonistic activity were subjected to assay for production of siderophore and the inhibitory effect of the culture filtrate on the pathogen.

3.2.6.2.1. Siderophore production

Selected isolates of bacteria, fungi and actinomycetes exhibiting antagonism under *in vitro* conditions were assayed for siderophore production in chrome azurol S (CAS) agar medium described by Schwyn and Neilands (1987). Test organisms were spot inoculated on CAS agar and incubated at 28 ± 2^0C for 7 days. Development of

yellow-orange halo around the growth was considered as positive for siderophore production. The control plates of CAS-agar (uninoculated) were incubated under the same conditions as described above and no color change was observed.

3.2.6.2.2. Effect of culture filtrate of antagonists on growth of P. capsici

Effect of culture filtrates of selected antagonists on the growth of *Phytophthora capsici* was studied as per the method given by Beena (2000).

To prepare the culture filtrate, 100ml potato dextrose broth in 250ml conical flasks was inoculated with loopful of bacterial and actinomycete antagonist. For fungal isolate, 5mm mycelia disc was inoculated. Culture filtrate was collected after 14 days of incubation for bacteria, whereas for actinomycetes and fungi, this was done after 21 days of incubation. The culture filtrate was passed through a sterile bacteriological filter (0.2µm pore size, Fisher Brand, Cat. No. 09-719C). Wells of 5mm diameter were cut using a cork borer at a distance of 2cm from the periphery in PDA plates supplemented with Sporidex 30ppm @ 100µl/100ml of the medium. Wells were then partially sealed with 75µl of molten soft agar. Then 100µl of the filtrate was carefully added into the wells using micropipette and was allowed to percolate. Five mm disc of *P. capsici* was placed at the centre of Petri plate. Plates were incubated at 28± 2^0C and three replications were maintained. Wells in the control plates were filled with potato dextrose broth instead of culture filtrate. Inhibition of mycelia growth of the pathogen was recorded after four days. Per cent inhibition over control was calculated as described in 3.2.5.3

3.2.6.3. Microscopic examination for hyphal interaction

Microscopic observation for hyphal interaction was taken by cutting out one sq. cm. portion of cellophane containing intermingling hyphal growth of antagonist and pathogen and mounting in cotton blue-lactophenol. Photomicrographs of mycoparasitism exhibited by the antagonist were taken.

3.2.6. *In planta* screening of selected isolates for antagonistic activity against *P. capsici* using black pepper as test crop

Three most promising antagonists including one isolate each of bacteria, actinomycete and fungus along with three most efficient PGPM selected from preliminary *in planta* screening were evaluated for their efficiency in controlling foot rot disease in black pepper cuttings. The experiment was conducted during June-August, 2014 at the Department of Agricultural Microbiology, College of Horticulture, Vellanikkara.

The treatment details of experiment were as follows

Design : CRD

Replications : 3*

Treatments : 10

Variety : Panniyur 1

Treatments:

T_1 : Selected PGPM-1 (nitrogen fixer)

T_2 : Selected PGPM-2 (phosphate solubiliser)

T_3 : Selected PGPM-3 (IAA producer)

T_4 : Selected antagonist-1 (bacteria)

T_5 : Selected antagonist-2 (actinomycete)

T_6 : Selected antagonist-3 (fungus)

T_7 : *Pseudomonas fluorescens* P1 (KAU reference culture)

T_8 : *Trichoderma viride* (KAU reference culture)

T_9 : Control with pathogen alone

T_{10} : Absolute control

* Each replication consisted of four polybags with four cuttings per bag.

3.2.6.1. Preparation of black pepper cuttings for in planta antagonistic study

Two month old rooted black pepper cuttings at two leaf stage, having uniform growth were selected for screening the efficiency of selected isolates against *P. capsici.* The seedlings were obtained from the Model Nursery on Spices, College of Horticulture, Vellanikkara.

3.2.6.2. Preparation and application of selected isolates

Inocula of five bacteria and actinomycete were prepared by inoculating loopful of individual colonies from agar plates in 200 ml of nutrient broth and Kenknigh't broth respectively and incubating at 28 ± 2^0C. Bacteria were incubated for 48h and actinomycetes for 5 days. In case of fungi, six mm discs were inoculated in 200 ml potato dextrose broth and incubated for 7days. After incubation the inocula were uniformly shaken and diluted to a concentration of 10^6 cfu ml^{-1} for bacteria and actinomycetes. For fungal isolates, dilution was carried out to obtain a spore concentration of 10^5 cfu ml^{-1}. Soil was inoculated with the microbial suspension at the rate of 30 ml per vine was given at the time of planting and 45 days after planting.

3.2.6.3. Mass multiplication and challenge inoculation of P. capsici

Discs were excised from PDA containing 7 day old culture of *P. capsici* and inoculated in 100g of sterile soil contained in a flask, along with 10 g of healthy black pepper leaves and incubated for one week. This soil was used as the source of *P. capsici* and applied @ 10g per vine, one month after the application of bioinoculants.

3.2.6.4. Biometric observations

Observations like length of vine, number of leaves, per cent disease incidence, per cent disease severity, foliar yellowing and biomass of seedlings were recorded.

3.2.6.4.1. Length of vine

The distance from the base of the seedling to the tip of the vine was taken as length of vine before and after 30, 60 and 90 days of last application of bioinoculants and expressed in centimeters.

3.2.6.4.2. Number of leaves

Total number of leaves per seedling was counted before and after 30, 60 and 90days of last application of bioinoculant and mean number of leaves were obtained.

3.2.6.4.3. Number of roots

Seedlings were removed from polybags 90 days after the last application of selected microbial isolates, and number of roots formed was counted and mean number obtained.

3.2.6.4.4. Length and fresh weight of roots

After 2 months of bioinoculant application, plants were uprooted and the length and fresh weight of roots were recorded. Length was expressed in cm and weight in gram.

3.2.6.4.5. Disease incidence

Incidence of *Phytophthora* foot rot in the nursery was recorded frequently after two weeks of challenge inoculation and the per cent disease incidence was calculated as follows

$$\text{Per cent disease incidence (PDI)} = \frac{\text{Number of cutting infected}}{\text{Total number of cuttings}} \times 100$$

3.2.6.4.6. Disease severity

The severity of disease on the leaves was recorded using a score chart based on 0-5 scale as shown in Table 2 and Plate 14.

$$\text{Per cent disease severity (PDS)} = \frac{\text{Sum of all numerical ratings}}{\text{Total number of leaves observed} \times \text{Maximum disease grade}} \times 100$$

Table 2. Score chart for severity of foot rot disease

Score	Leaf area infection
0	No infection
1	< 10 %
2	>10% - 25%
3	>25% - 50 %
4	>50% - 75 %
5	>75% or defoliated or death of vine

3.2.6.4.7. Foliar yellowing

Number of vines showing symptoms of foliar yellowing was recorded and per cent foliar yellowing of vine was calculated. For intensity of foliar yellowing, grades were given based on visual observation using following scale given in Table 3.

Table 3. Score chart for intensity of foliar yellowing

Score	Foliar yellowing
0	No symptom
1	Mild
2	Moderate
3	Severe

3.2.7. Identification of selected PGPM and antagonists

The selected PGPM and antagonists were identified based on cultural, morphological and biochemical characters.

3.2.7.1. 16s rDNA sequence analysis of bacterial and actinomycetes

After *in vitro* and *in planta* screening for plant growth promotion and antagonistic activities, six most promising bacteria and actinomycetes were identified by 16S rDNA sequence analysis.

3.2.7.1.1. Amplification of 16S rDNA gene

A colony was taken using microtip, mixed with 10 µl sterile water. Two µl of the culture suspension was used as template for amplification of 16S rDNA gene. The details of primer used are given in Table 4.

Polymerase chain reaction was carried out in Eppendorf Master Cycler (Gradient) using PCR master mix 'Emerald Amp GT PCR'. The composition of the reaction mixture for PCR are given in Table 5.

The reaction was set in 200 µl microfuge tube chilled over ice flakes. A momentary spin was given to mix completely all reagents and set in master cycler for amplification. The details of master cycler programme are detailed in Table 6.

The quality of isolated DNA was evaluated though agarose gel electrophoresis (Sambrook *et al.*, 1989). 1X TAE buffer was prepared from the 50X TAE (pH 8.0) stock solutions. Agarose (Genei, Low EEO) (1%) was weighed and dissolved in TAE buffer by boiling. Ethidium bromide prepared from a stock of 10 mg ml^{-1} was added to it at a concentration of 0.5 µg ml^{-1} and mixed well. The comb was placed properly and dissolved agarose was poured into the tray. The gel was placed in the electrophoresis unit after 30 min with well side directed towards the cathode. 1X TAE buffer was added to the buffer tank (Genei, Bangalore) so as to cover the well with a few millimeter of buffer. 2 µl of the PCR product was carefully loaded into the wells using a micro pipette. The Gene Ruler 1 kb DNA ladder was used as the molecular weight marker. The cathode and anode of the electrophoresis unit were connected to the power pack and the gel was run at constant voltage of 100 V. The power was turned off when the tracking dye reached at about 3 cm from the anode end.

Table 4. Details of primers used for 16S rDNA gene amplification

Primer details	**Sequence 5'- 3'**	**Base pair**
8 F	AGAGTTTGATCCTGGCTCAG	20
1522 R	AAG GAG GTG ATC CAG CCG CA	20

Table 5. Composition of PCR reaction mixture

Component	**Per reaction volume required**
Master mix	12.5 µl
Template	2.0 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
dH_2O	9.5 µl
Total	25.0 µl

Table 6. Details of master cycle programme

No.	Step	Temperature (^{0}C)	Time (min)
1	Initial denaturation	95	3.00
2	Denaturation	94	1.30
3	Annealing	55	0.40
4	Primer extension	72	01.30
5	Steps 2-4	34 cycles	-
6	Final extension	72	20.00
7	Final hold	4	10.00

3.2.7.1.2. Gel documentation

The DNA bands separated by electrophoresis were viewed and photographed using gel documentation imaging system.

3.2.7.1.3. Purification and sequencing of PCR product

The PCR product was purified and sequenced at Scigenom Pvt. Ltd. Cochin, using the primers 8F and 1522R.

3.2.7.1.4. Nucleotide sequence analysis

The blastn programme (http://blast.ncbi.nlm.nih.gov/Blast.) was used to find out the homology of the nucleotide sequences. The National Center for biotechnology Information (NCBI) accession sharing maximum homology with the query sequence was used to identify the isolate.

3.2.7.2. Identification of selected fungal isolates

Morphological and cultural characters of the selected fungal isolates were studied in PDA. Hyphal morphology and presence of spores were studied under 100X (oil immersion) of compound microscope and photographs taken. Confirmation of identification was carried out at National Centre for Fungal Taxonomy (NCFT), New Delhi.

3.2.8. Statistical analysis

Analysis of variance was done on the data collected using the statistical package MSTAT (Freed, 1986). Multiple comparisons among the treatment means were done using DMRT.

Results

4. RESULTS

The results of the investigation entitled "Functional diversity of beneficial microorganisms from the rhizosphere of black pepper in Wayanad" undertaken during the period 2012-2014 at Department of Agricultural Microbiology, are presented below.

4.1. COLLECTION OF RHIZOSPHERE SOIL SAMPLES FROM WAYANAD DISTRICT

Twelve rhizospheric soils were collected from black pepper gardens from different locations of Wayanad district (Plate 1). These 12 samples comprised four each from healthy, foot-rot affected and yellowing affected pepper gardens. Details of soil samples collected along with geographical positions are given in Table 7.

4.2. POPULATION OF RHIZOSPHERE MICROFLORA

Population of bacteria, fungi, actinomycetes and beneficial microorganisms such as nitrogen fixers, phosphate solubilizers, fluorescent pseudomonads and *Bacillus* were estimated in rhizosphere soils collected from Wayanad district (Table 8) (Plate 2-3)

Among the different samples analysed, HPR recorded highest population of bacteria ($16.6x10^6$cfu g^{-1}) followed by HVZ ($8.3x10^6$cfu g^{-1}). Lowest bacterial population was observed in FMY ($0.2x10^6$cfu g^{-1}). In general, healthy rhizosphere soils with exception of HAB recorded higher bacterial population than disease affected rhizosphere soils.

Healthy rhizosphere soils except HAB and HVZ recorded highest fungal population than the rhizosphere soils from disease affected gardens. Among the healthy rhizosphere soils HPL recorded highest fungal population ($6.0x10^4$cfu g^{-1}).

In case of actinomycetes, rhizosphere soils from yellowing affected garden with exception of YPT recorded highest population followed by healthy gardens. Least population of actinomycetes was noticed in foot rot affected gardens. Among the yellowing affected ones YPR recorded highest population of actinomycetes (5.2 x 10^4cfu g^{-1}) followed by YPL (4.8 x 10^4cfu g^{-1}).

Rhizosphere soils from the yellowing affected gardens recorded highest population of nitrogen fixers followed by healthy rhizosphere soils. Among the yellowing affected gardens YAB recorded highest population of nitrogen fixers (7.3 x 10^4cfu g^{-1}) followed by YPT (5.3 x 10^4cfu g^{-1}).

Compared to foot rot and yellowing affected gardens, healthy garden HPL recorded highest population of phosphate solubilizers (9.3 x 10^5cfu g^{-1}), Fluorescent pseudomonads (2.5 x 10^3cfu g^{-1}). In case of *Bacillus* yellowing affected garden YPR recorded highest population (3.4x10^6cfu g^{-1}) followed by healthy garden HPL (2.8 x 10^6cfu g^{-1}).

4.3. PURIFICATION AND MAINTENANCE OF ISOLATES

A total of 207 predominant rhizosphere isolates, comprising 38 nitrogen fixers, 24 P-solubilisers, 20 *Bacillus*, 32 actinomycetes, 63 fungi, 5 fluorescent pseudomonads and 25 other bacteria were purified and maintained as per standard procedures

Pulpalli

Vazhakavala

A. Healthy

Perikallur

Pathiri

B. Foot rot affected

Pulpulli

Perikallur

C. Yellowing affected

Plate 1: Black pepper gardens in Wayanad selected for collection of rhizosphere soil

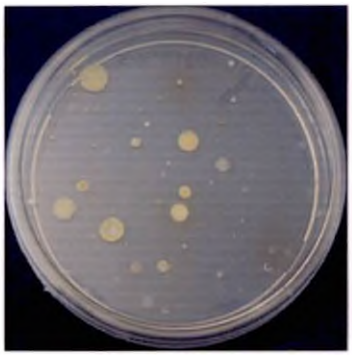
Table 7. Details of soil samples collected from the rhizosphere of black pepper in Wayanad

Location	Code	Geographical position			Standard
		Latitude (N)	Longitude (E)	Altitude (ft)	
Healthy gardens					
Perikallur	HPR	$11^{0}85.875'$	$76^{0}16.554'$	2445	*Glyricidia*
Pulpulli	HPL	$11^{0}85.686'$	$76^{0}18.686'$	2481	*Glyricidia*
Ambalavayal	HAB	$11^{0}61.062'$	$76^{0}21.093'$	3072	Silver oak
Vazhakavala	HVZ	$11^{0}83.589'$	$76^{0}17.632'$	2626	Rubber
Foot rot affected gardens					
Perikallur	FPR	$11^{0}85.895'$	$76^{0}15.160'$	2430	*Glyricidia*
Pulpulli	FPL	$11^{0}85.686'$	$76^{0}18.686'$	2481	*Erythrina*
Pathiri	FPT	$11^{0}84.669'$	$76^{0}14.649'$	2445	*Glyricidia*
Mayilumbadi	FMY	$11^{0}69.891'$	$76^{0}17.333'$	2572	*Erythrina*
Yellowing affected gardens					
Perikallur	YPR	$11^{0}85.895'$	$76^{0}15.160'$	2430	*Glyricidia*
Pulpulli	YPL	$11^{0}85.686'$	$76^{0}18.686'$	2481	*Erythrina*
Pathiri	YPT	$11^{0}84.669'$	$76^{0}14.649'$	2445	*Glyricidia*
Ambalavayal	YAB	$11^{0}61.062'$	$76^{0}21.093'$	3072	Silver oak

H-Healthy F-Foot rot Y-Yellowing

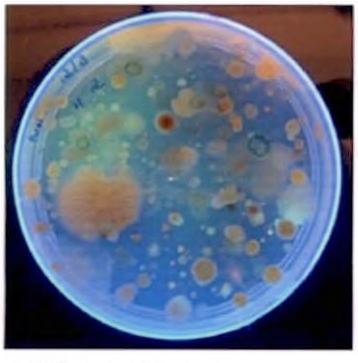
N-North E-East ft- Feet

Nutrient agar

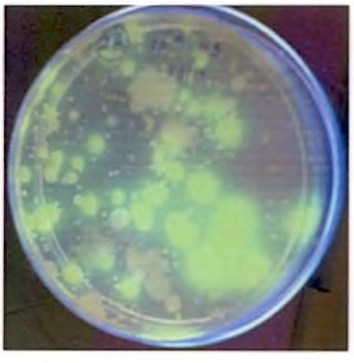


Oligotrophic nutrient agar

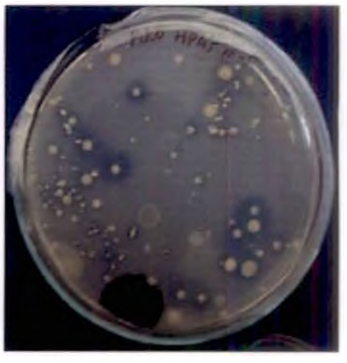
Luria agar



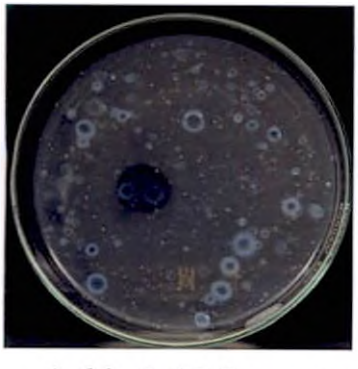
Kings A agar



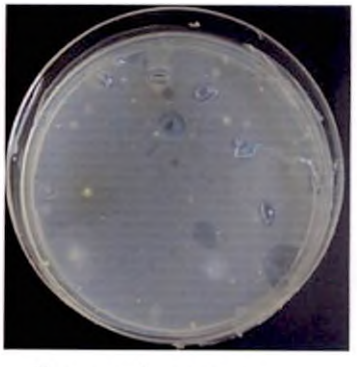
Kings B agar



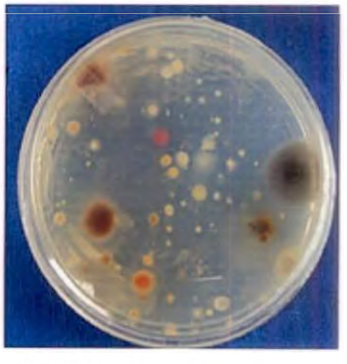
Pikovskaya's agar



Ashby's N free agar

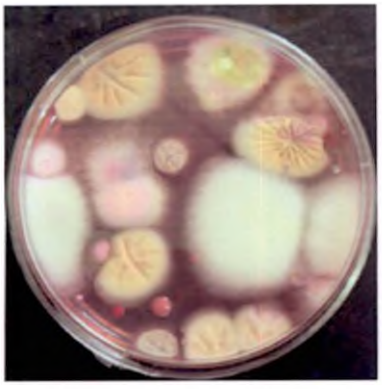


Jensen's agar

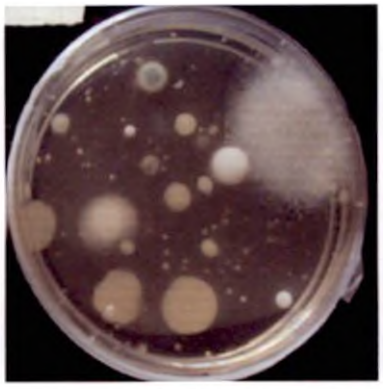


Soil extract agar

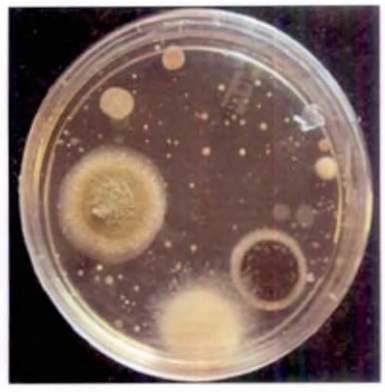
Plate 2 :Rhizosphere microflora isolated on different media



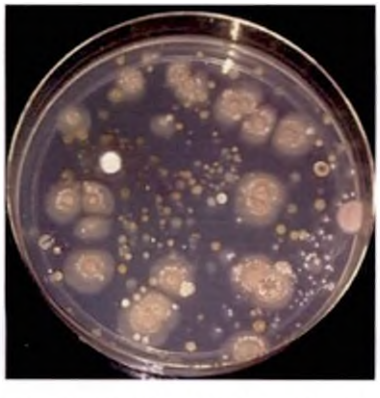
Rose Bengal agar



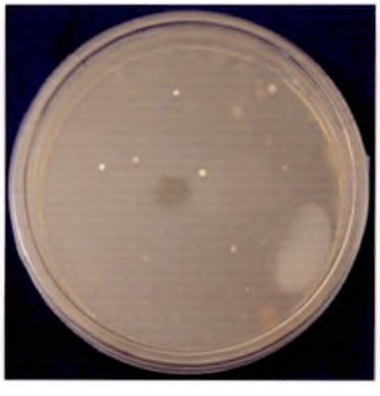
Potato dextrose agar



Oligotrophic Potato dextrose agar



Kenknight & Munaier's agar



Oligotrophic Kenknight & Munaier's agar

Kuster's agar

Plate 3. Rhizosphere microflora isolated on different media

Table 8. Population of rhizosphere microflora in black pepper gardens of Wayanad

Location	Code	Population of microorganisms (cfu per gram of soil)						
		Bacteria x 10^6	Fungi x 10^4	Actinomycetes x 10^3	Nitrogen fixers x 10^4	Phosphate solubilizers x 10^5	*Pseudomonas* x 10^3	*Bacillus* x 10^6
Healthy gardens								
Perikallur	HPR	16.6	5.3	2.7	3.8	2.7	1.2	1.0
Pulpalli	HPL	3.9	6.0	3.2	4.0	9.3	2.5	2.8
Ambalavayal	HAB	1.2	1.8	1.7	2.5	4.7	1.3	1.4
Vazhakavala	HVZ	8.3	3.2	3.8	5.0	1.7	1.5	0.5
Foot rot affected gardens								
Perikallur	FPR	0.5	3.8	1.5	2.0	3.7	1.0	0.5
Pulpalli	FPL	2.1	3.2	0.3	2.7	1.3	1.8	1.5
Pathiri	FPT	0.6	1.7	2.5	1.1	3.3	0.7	0.3
Mayilumbadi	FMY	0.2	5.2	1.5	3.7	2.0	1.2	1.3
Yellowing affected gardens								
Perikallur	YPR	2.4	3.7	5.2	4.5	8.3	1.0	3.4
Pulpalli	YPL	1.1	2.8	4.8	2.8	3.0	1.7	1.0
Pathiri	YPT	1.4	3.0	2.5	5.3	0.4	1.3	1.9
Ambalavayal	YAB	1.4	2.8	1.8	7.3	2.3	1.0	0.8

H - Healthy F - Foot rot Y – Yellowing

4.4. *IN VITRO* SCREENING FOR PLANT GROWTH PROMOTING (PGP) ACTIVITIES

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

All the 207 isolates were screened for the IAA production *in vitro*. Based on the development of pink to red color, ten isolates (HPLBC-6, HABC-3, HPLPSB-3, HALN-4, HPLPSB-4, HVKN-2, HPRPSB-1, FPTPF-1, FMYBC-2 and YPTN-3) were found to be positive for IAA production (Plate 4A) Amount of IAA produced by these isolates were further quantified.

4.4.1.1. Quantitative estimation of indole acetic acid (IAA)

In vitro auxin production by ten selected isolates was quantitatively estimated and the data are in Table 9 (Plate 4B). IAA production ranged from 4.15 μg ml^{-1} to 292.50 μg ml^{-1}. The isolate HPLBC-6 was found to be highest IAA producer with a production of 292.50 μg ml^{-1}, which was significantly different from all the other isolates. It was followed by HABC-3 with 46.43 μg ml^{-1} and HPLPSB-3 with 24.02 μg ml^{-1}. The least IAA production of 4.15 μg ml^{-1} was recorded by isolate HPRPSB-1.

4.4.2. Screening for phosphate solubilization

4.4.2.1. Screening for phosphate solubilizing activity

Twenty four isolates obtained were screened for P solubilization in Pikovskaya's agar media (Plate 5). Among these, ten isolates were found to be positive for P solubilization and the amount of P solubilized was further quantified as indicated in Table 4.

4.4.2.2. Quantitative estimation of phosphate solubilization

Among the ten isolates found to be solubilizing phosphate, quantity of P solubilized was found to be highest by the isolate HPLPSB-3 (from healthy garden) with 162.7 μg $ml^{-1}$and this was followed by HPLF-5 (from healthy garden) with 161.3 μg ml^{-1}. A corresponding reduction in pH was noticed indicating the production of organic acids (Table 11).

4.4.3. Screening for nitrogen fixation in nitrogen free medium

A total of 38 nitrogen fixers were screened for their nitrogen fixation activity based on their growth in nitrogen free medium (Table 12). Among these, three isolates from healthy gardens viz. HPLN-3, HVKN-6, HVKN-7 and two isolates from yellowing affected gardens (YPTN-3 and YPRN-3) showed excellent growth on nitrogen free medium. Five isolates (HPRN-1, HPRN-2, HPRN-5, HVKN-4 and HVKN-5) from healthy gardens, four isolates (FPLN-3, FPTN-4, FMLN-3 and FMLN-4) from foot rot affected gardens and four isolates (YPRN6, YPTN-1, YPTN-2 and YPTN-4) from yellowing affected gardens showed good growth on nitrogen free medium. Fourteen showed moderate growth and rest of 6 isolates were categorized as poor nitrogen fixers. Five isolates which recorded excellent growth on N-free medium were further selected for quantification of fixed N under *in vitro* conditions (Plate 6).

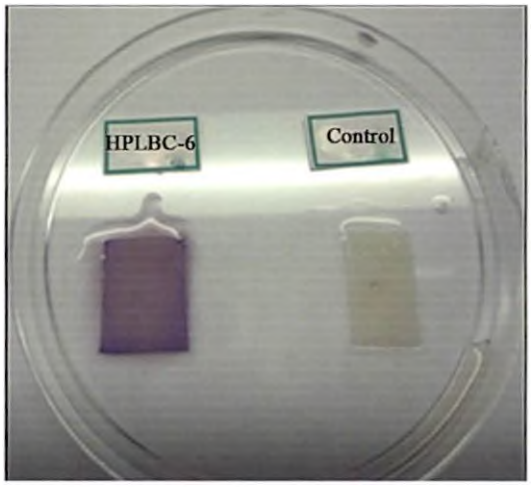
4.4.3.1. Estimation of nitrogen fixed by micro-Kjeldhal method

Amount of nitrogen fixed by the selected five excellent nitrogen fixers was estimated by micro-Kjeldhal method. Among the five isolates, YPTN-3 from yellowing affected garden fixed maximum amount of nitrogen (46.92 mg) which was on par with the isolate HVKN-6 (32.62 mg) obtained from healthy garden.

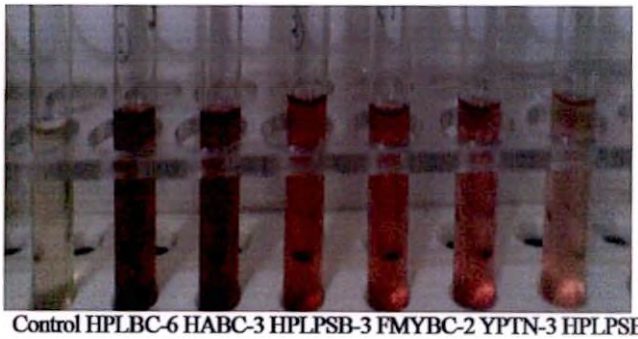
Table 9. Quantitative estimation of indole acetic acid

Sl. No.	Isolates	Concentration of IAA (µg ml^{-1})
1	HPLBC-6	292.50 (17.11^{a})*
2	HABC-3	46.43 (6.84^{b})
3	HPLPSB-3	24.02 (4.95^{c})
4	HALN-4	6.10 (2.56^{g})
5	HPLPSB-4	8.14 (2.94^{f})
6	HVKN-2	5.23 (2.39^{gh})
7	HPRPSB-1	4.15 (2.15^{h})
8	FPTPF-1	14.77 (3.90^{e})
9	FMYBC-2	22.58 (4.80^{cd})
10	YPTN-3	19.88 (4.51^{d})

* Square root transformed values are given in parentheses



A. Qualitative screening



Control HPLBC-6 HABC-3 HPLPSB-3 FMYBC-2 YPTN-3 HPLPSB-4

B. Quantitative estimation

Plate 4. Sreening for IAA production

Table 10. Screening for phosphate solubilization

Sl. No	Isolates	Formation of clear zone in Pikovskaya's agar medium	Solubilization index
1	HPRPSB-1	+	184.1
2	HPRPSB-2	+	184.9
3	HPRPSB-3	-	-
4	HPLPSB-1	+	-
5	HPLPSB-3	+	216.7
6	HPLF-1	+	102.7
7	HPLF-5	+	120.6
8	HABPSB-1	-	-
9	HABPSB-2	+	126.0
10	FMYPSB-1	-	-
11	FMYPSB-2	+	121.6
12	FPTPSB-1	+	152.7
13	FPTPSB-2	-	-
14	FPTPSB-3	-	-
15	FPTPSB-4	+	126.0
16	FPRPSB-3	-	-
17	FPRPSB-4	-	-
18	YPTPSB-1	+	127.8
19	YPRPSB-1	-	-
20	YPLPSB-2	-	-
21	YPLPSB-3	-	-
22	YPLPSB-4	-	-
23	YABPSB-1	-	-
24	YABPSB-2	-	-

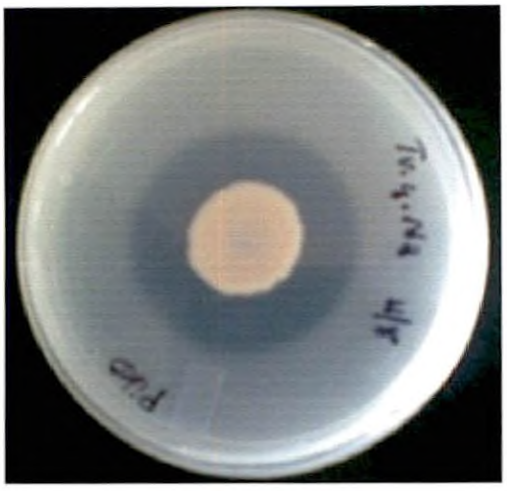
+ indicates clear zone formation in Pikovskaya's medium

- absence of clear zone

Table 11. Quantitative estimation of Phosphate solubilization by selected isolates

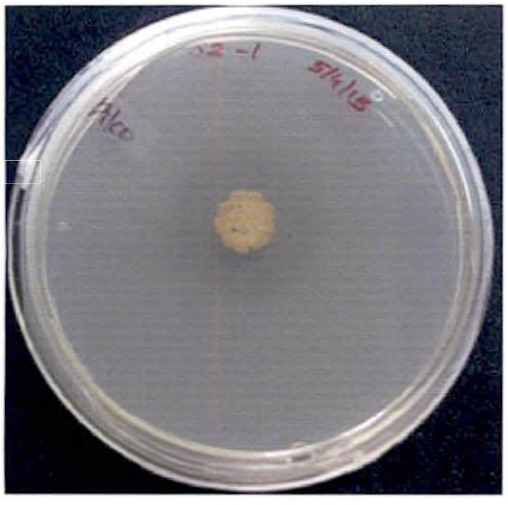
Isolates	**Quantity of P-solubilized (µg ml-1)**	**Reduction in pH***
HPRPSB-1	138.7[c]	4.4
HPRPSB-2	85.60[g]	5.7
HPLPSB-3	162.7[a]	3.4
HABPSB-2	134.9[d]	4.5
FMYPSB-2	72.00[h]	5.8
FPTPSB-1	87.20[g]	5.7
FPTPSB-4	147.2[b]	3.6
YPTPSB-1	94.93[f]	5
HPLF-1	98.80[e]	5.2
HPLF-5	161.3[a]	3.4

* Initial pH was adjusted to 7.2

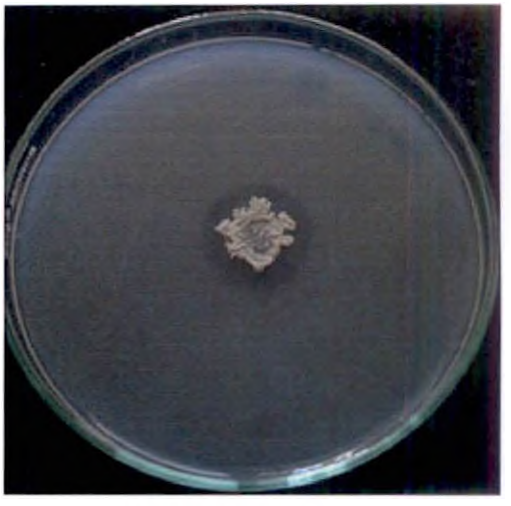


HPLPSB-3

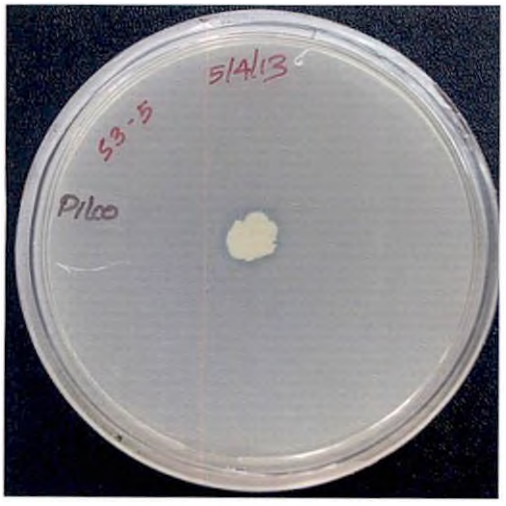
HPLF-1



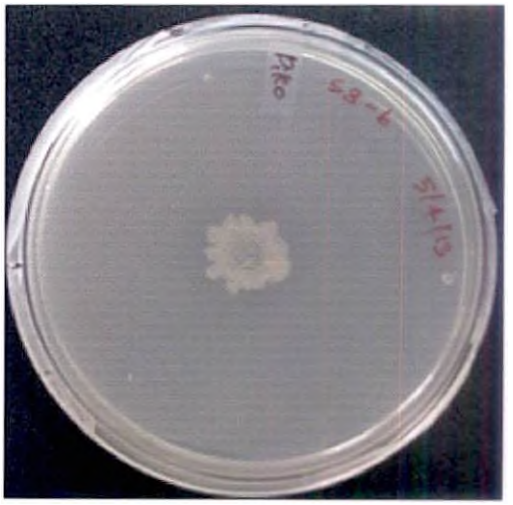
FTPSB-4



HPRPSB-1



HABPSB-2



YPTPSB-1

Plate 5. Phosphate solubilisation by microbes on Pikovskaya's agar medium

Table 12. Screening of nitrogen fixers for nitrogen fixation

Sl. No.	Isolates	Growth on N free medium*
1	HPRN-1	+++
2	HPRN-2	+++
3	HPRN-3	+
4	HPRN-4	++
5	HPRN-5	+++
6	HPLN-1	+
7	HPLN-2	++
8	HPLN-3	++++
9	HPLN-4	++
10	HPLN-5	+
11	HALN-1	++
12	HALN-2	++
13	HALN-3	+
14	HALN-4	++
15	HALN-5	++
16	HVKN-1	++
17	HVKN-2	+
18	HVKN-3	++
19	HVKN-4	+++
20	HVKN-5	+++
21	HVKN-6	++++
22	HVKN-7	++++
23	FMLN-3	+++
24	FMLN-4	+++

Table 13.Quantity of nitrogen fixed by selected isolates under *in vitro* conditions

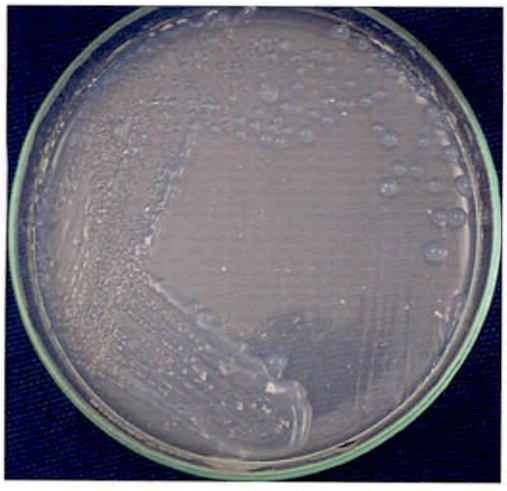
Isolates	**Amount of nitrogen fixed (mg of N g^{-1} of sucrose utilized)***
HPLN-3	23.57^{bc}
HVKN-6	32.62^{ab}
HVKN-7	19.21^{c}
YPTN-3	46.92^{a}
YPRN-3	10.94^{c}

* Mean of three replications

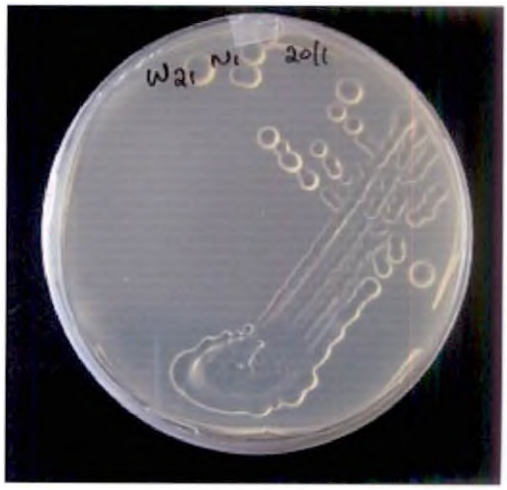
HPLN-3

HVKN-7

YPTN-3



YPRN-3



HVKN-6

Plate 6. Nitrogen fixing bacteria on N free Jensen's agar

4.5. EVALUATION OF SELECTED PGPM FOR GROWTH PROMOTION IN BLACK PEPPER CUTTINGS

Six most efficient PGPM including two nitrogen fixers, two phosphate solubilisers and two IAA producers were evaluated for plant growth promotion in black pepper cuttings.

Treatments:

T_1 : Selected nitrogen fixer I (YPTN-3; *Paenibacillus* sp.)

T_2 : Selected nitrogen fixer II (HVKN-6; *Paenibacillus* sp.)

T_3: Selected phosphate solubiliser I (HPLPSB-3; *Acinetobacter grimontii*)

T_4 : Selected phosphate solubiliser II (HPLF-5; *Gongoronella butleri*)

T_5 : Selected IAA producer I (*Providencia* sp.)

T_6 : Selected IAA producer II (*Providencia* sp.)

T_7 : *Pseudomonas fluorescens*-P1 (KAU reference culture)

T_8 : Absolute control

4.5.3. Biometric observations

4.5.3.1. Sprouting

After 30 days of planting, T_3 (HPLPSB-3) recorded highest germination of 32.82 per cent. This was followed by T_1 (YPTN-3), which recorded a germination of 33.33 per cent and these two were on par with all other treatments except T_2 (HVKN-6) and T_8 (Absolute control).

After 60 and 90 days of planting, maximum sprouting was recorded by T_3 (HPLPSB-3). Minimum sprouting (52.00 %) after 90 days of planting was observed in T_8 (Absolute control). Data showing the results are given in Table 14.

4.5.3.2. Length of vine

Effect of PGPM on the length of the pepper vines are given in Table 15, Plate 7. After 120 days of planting, T_3 (HPLPSB-3) recorded maximum shoot length of 13.53 cm. This was statistically on par with T_7 (*Pseudomonas fluorescens* [KAU]), T_2 (HVKN-6) and T_4 (HPLF-5) with 12.50cm, 11.96cm and 11.59cm respectively. Minimum length of 8.46cm was observed in T_6 (HABC-3).

Similar trend was observed six months after planting and T_3 (HPLPSB-3,) recorded maximum length of 36.02 cm. This was statistically on par with T_4 (HPLF-5) and T_7 (*Pseudomonas fluorescens* [KAU]). Minimum shoot length of 17.43cm was observed in T_8 (Absolute control).

4.5.3.3. Number of leaves

Effect of PGPM on the number of leaves is shown in Table 16, Plate 7. No significant differences were noticed intially after two months of planting. After four months of planting T_3 (HPLPSB-3) was found to be statistically superior to others with 3.63 leaves. Minimum number of leaves (1.50) was observed in T_5 (HPLBC-6). This was followed by T_8 (Absolute control) with 1.83 leaves.

After six months of planting, T_3 (HPLPSB-3) was significantly superior than other treatments and on par with the treatment T_4 (HPLF-5).

Table14. Effect of PGPM on sprouting of pepper cuttings

Treatments		Sprouting per treatment (%)		
		30 DAP *	60 DAP *	90 DAP*
T_1	YPTN-3: *Paenibacillus* sp.	33.33 (5.81^{a})	37.50 (6.16^{ab})	68.75^{b}
T_2	HVKN-6: *Paenibacillus* sp.	20.83 (4.57^{b})	29.17 (5.39^{b})	54.16^{c}
T_3	HPLPSB-3: *Acinetobacter grimontii*	32.82 (5.76^{a})	46.88 (6.82^{a})	83.33^{a}
T_4	HPLF-5: *Gongronella butleri*	25.00 (5.02^{ab})	46.88 (6.89^{a})	56.25^{c}
T_5	HPLBC-6: *Providencia* sp.	28.13 (5.30^{ab})	39.58 (6.25^{ab})	56.25^{c}
T_6	HABC-3: *Providencia* sp.	21.88 (4.72^{ab})	28.13 (5.35^{b})	80.20^{ab}
T_7	*Pseudomonas fluorescens* (KAU)	29.17 (5.44^{ab})	56.25 (7.53^{a})	80.20^{a}
T_8	Absolute control	18.75 (4.35^{b})	29.17 (5.44^{b})	52.00^{c}

Mean of three replications

DAP - Days after planting

Table 15. Effect of PGPM on length of pepper vine

Treatments		Length of pepper vine (cm)	
		120 DAP	150 DAP
T_1	YPTN-3: *Paenibacillus* sp.	8.54^{b}	22.49^{bc}
T_2	HVKN-6: *Paenibacillus* sp.	11.96^{ab}	20.53^{bc}
T_3	HPLPSB-3: *Acinetobacter grimontii*	13.53^{a}	36.02^{a}
T_4	HPLF-5: *Gongronella butleri*	11.59^{ab}	28.74^{abc}
T_5	HPLBC-6: *Providencia* sp.	8.46^{b}	18.48^{c}
T_6	HABC-3: *Providencia* sp.	9.04^{b}	20.88^{bc}
T_7	*Pseudomonas fluorescens* (KAU)	12.50^{ab}	32.33^{ab}
T_8	Absolute control	8.84	17.43^{c}

DAP- Days after planting

Table 16. Effect of PGPM on number of leaves

Treatments		30 DAP *	120 DAP	150 DAP
T_1	YPTN-3: *Paenibacillus* sp.	1.00	1.61^{ab}	3.56^{c}
T_2	HVKN-6: *Paenibacillus* sp.	1.53	1.72^{ab}	3.50^{c}
T_3	HPLPSB-3: *Acinetobacter grimontii*	1.67	1.97^{a}	6.50^{a}
T_4	HPLF-5: *Gongronella butleri*	1.12	1.21^{ab}	4.11^{bc}
T_5	HPLBC-6: *Providencia* sp.	1.00	1.09^{b}	3.17^{c}
T_6	HABC-3: *Providencia* sp.	1.21	1.47^{ab}	5.33^{ab}
T_7	*Pseudomonas fluorescens* (KAU)	1.47	1.32^{ab}	3.83^{bc}
T_8	Absolute control	1.00	1.06^{b}	3.67^{bc}

*Significantly not different

DAP –Days after planting

Control *Paenibacillus* sp.

Paenibacillus sp. Control

Acinetobacter grimontii Control

Gongronella butleri Control

Providencia sp. Control

Providencia sp. Control

Control *Pseudomonas fluorescens*

Plate 7. Effect of PGPM on plant growth

Table 17. Effect of PGPM on root parameters

Treatments		Growth parameters per vine			
		Mean number of roots	**Mean length of roots (cm)**	**Fresh weight of roots (g)**	**Dry weight of roots (g)***
T_1	YPTN-3: *Paenibacillus* sp.	5.75^{bc}	13.48^{ab}	0.07 (0.75^{b})	0.03 (0.73)
T_2	HVKN-6: *Paenibacillus* sp.	11.00^{a}	13.50^{ab}	0.16 (0.81^{ab})	0.06 (0.75)
T_3	HPLPSB-3: *Acinetobacter grimontii*	10.12^{a}	14.79^{ab}	0.35 (0.91^{a})	0.09 (0.77)
T_4	HPLF-5: *Gongronella butleri*	9.50^{ab}	14.42^{ab}	0.15 (0.81^{ab})	0.08 (0.76)
T_5	HPLBC-6: *Providencia* sp.	9.00^{abc}	19.13^{a}	0.23 (0.85^{ab})	0.05 (0.74)
T_6	HABC-3: *Providencia* sp.	8.00^{abc}	11.50^{b}	0.11 (0.78^{ab})	0.08 (0.76)
T_7	*Pseudomonas fluorescens* (KAU)	5.20^{c}	11.49^{b}	0.07 (0.76^{b})	0.05 (0.74)
T_8	Absolute control	8.00^{abc}	10.00^{b}	0.05 (0.75^{b})	0.03 (0.73)

4.5.3.4. Number of roots

Maximum number of roots (11.00) were observed in T_2 (HVKNF-6). This was followed by T_3 (HPLPSB-3) with 10.12 roots. Minimum number of roots (5.20) were observed in T_7 (*Pseudomonas fluorescens* [KAU]). The data is given in Table 17, Plate 8.

4.5.3.5. Length of roots

Maximum root length (19.13cm) was observed in T_5 (HPLBC-6) and this was found to be statistically superior to all other treatments (Plate 8). All other treatments except T_6 (HABC-3), T7 (*Pseudomonas fluorescens* [KAU]) and T_8 (Absolute control) were found to be statistically on par with each other Table 17.

4.5.3.6. Fresh weight of roots

Fresh weight of roots was found to be maximum (0.35g) in T3 (HPLPSB-3, from healthy garden) and this was found to be statistically superior to all other treatments. Minimum fresh weight of roots (0.05g) was observed in T8 (Absolute control). Table 17.

4.5.3.7. Dry weight of roots

Data on the dry weight of roots revealed that there was no significant difference among the treatments.

Table 17. Effect of PGPM on root parameters

Treatments		Growth parameters per vine			
		Mean number of roots	**Mean length of roots (cm)**	**Fresh weight of roots (g)**	**Dry weight of roots (g)***
T_1	YPTN-3: *Paenibacillus* sp.	5.75^{bc}	13.48^{ab}	0.07 (0.75^{b})	0.03 (0.73)
T_2	HVKN-6: *Paenibacillus* sp.	11.00^{a}	13.50^{ab}	0.16 (0.81^{ab})	0.06 (0.75)
T_3	HPLPSB-3: *Acinetobacter grimontii*	10.12^{a}	14.79^{ab}	0.35 (0.91^{a})	0.09 (0.77)
T_4	HPLF-5: *Gongronella butleri*	9.50^{ab}	14.42^{ab}	0.15 (0.81^{ab})	0.08 (0.76)
T_5	HPLBC-6: *Providencia* sp.	9.00^{abc}	19.13^{a}	0.23 (0.85^{ab})	0.05 (0.74)
T_6	HABC-3: *Providencia* sp.	8.00^{abc}	11.50^{b}	0.11 (0.78^{ab})	0.08 (0.76)
T_7	*Pseudomonas fluorescens* (KAU)	5.20^{c}	11.49^{b}	0.07 (0.76^{b})	0.05 (0.74)
T_8	Absolute control	8.00^{abc}	10.00^{b}	0.05 (0.75^{b})	0.03 (0.73)

T1- *Paenibacillus* sp.

T2- *Paenibaillus* sp.

T3- *Acinetobacter grimontii*

T4- *Gongronella butleri* T5- *Providencia* sp. T7- *P. fluorescens* T8-Control

Plate 8. Effect of PGPM on root growth parameters

4.6. Screening of beneficial microorganisms for antagonistic activity on *Phytophthora capsici*

4.6.1. Isolation and identification of pathogen

The pathogen *P. capsici* was successfully isolated on carrot agar medium from the foot rot affected black pepper leaves as explained as in materials and method. Pathogen was then purified and identified by its cultural and morphological characters (Plate 9A).

4.6.2 Test for pathogenicity

Pepper leaves inoculated with mycelial disc of *P. capsici* were developed water soaked lesions after 48 hours of incubation (Plate 9B). The lesions were black, circular in shape which enlarged and covered the entire leaf surface upon incubation.

4.6.3 Screening of bacterial isolates against *P. capsici*

A total of 112 bacterial isolates were screened for their antagonistic activity against *P. capsici* by dual culture method. Per cent inhibition was calculated after seven days of incubation (Table 18, Plate10). Based on their per cent inhibiton isolates were ranked. Among 112 bacterial isolates, two isolates HPLPSB-6 and HABB-1 obtained from healthy pepper gardens scored first rank. Among these HPLPSB-6 gave 69.27 per cent inhibition followed by HABB-1 with 65.00 per cent.

Five isolates, HPLPSB-3 (60.00), HABBC-2 (60.00), HABBC-1 (53.66), obtained from healthy gardens and FPTPF-2 (56.10), FPRPF-3 (56.09) from foot rot affected gardens scored the second rank.

Ten isolates, HABB-2 (50.00), HPLPSB-2 (49.22), HPLPF-2 (47.05), HPLB-2 (45.00), HPRB-1 (43.43), obtained from healthy gardens,FPTB-2 (45.00),FPRPSB-3 (43.11), FMLPSB-2 (42.00), obtained from foot rot affected gardens and YPTBC-2

A. *P. capsici* isolated from infected black pepper leaves

B. Artificially infected Black pepper leaves with *P. capsici*

C. Formation of water soaked lesions

Plate 9. *P.capsici* isolated from infected leaf and artificial infestation in Black pepper leaves

(48.78), YPLPSB-4 (42.00) obtained from yellowing affected garden were scored the third rank.

Four isolates were scored the fourth rank. The isolates were HPLPSB-5 (36.50), HPLBC-5 (32.35) from healthy gardens, FPTPSB-2 (36.12) from foot rot affected garden and YPRBC-2 (32.35) from yellowing affected garden.

Fourteen isolates including HPLPSB-4 (26.83), HPRB-6 (26.83),HALPSB-1 (26.82), HPRPSB-1 (26.12), HPRPSB-2 (23.43), HPLB-1 (21.95) obtained from healthy gardens, FMYBC-1 (26.83), FPRBC-3 (26.83), FPTPSB-4 (26.83) obtained from foot rot affected gardens and YALPSB-2 (26.83), YPRBC-1 (26.82), YPRPSB-1 (22.93), YPLBC-1 (21.95), YPLPSB-3 (21.34), obtained from yellowing affected gardens were scored the fifth rank.

About twenty five isolates were scored the sixth rank and rest of the 52 isolates were fell under last group with seventh rank.

Table 18. Screening of bacterial isolates against *P. capsici*

Sl. No	Isolates	Per cent inhibition	Rank
1	HPRN-1	4.88	7
2	HPRN-2	2.44	7
3	HPRN-3	0.00	7
4	HPRN-4	0.00	7
5	HPRN-5	0.00	7
6	HPLN-1	0.00	7
7	HPLN-2	0.00	7
8	HPLN-3	0.00	7
9	HPLN-4	7.32	7
10	HPLN-5	0.00	7
11	HALN-1	2.44	7
12	HALN-2	0.00	7
13	HALN-3	0.00	7
14	HALN-4	14.63	6
15	HALN-5	0.00	7
16	HVZN-1	0.00	7
17	HVZN-2	14.63	6
18	HVZN-3	0.00	7
19	HVZN-4	12.20	6
20	HVZN-5	0.00	7
21	HVZN-6	9.76	7
22	HVZN-7	2.44	7
23	FMLN-3	0.00	7
24	FMLN-4	0.00	7
25	FMLN-5	0.00	7
26	FPTN-3	4.88	7
27	FPTN-4	0.00	7
28	FPLN-2	0.00	7
29	FPLN-3	0.00	7

30	YPTN-1	0.00	7
31	YPTN-2	2.44	7
32	YPTN-3	7.32	7
33	YPTN-4	12.20	6
34	YPRN-3	0.00	7
35	YPRN-6	0.00	7
36	YALN-2	0.00	7
37	YALN-3	0.00	7
38	YALN-4	0.00	7
39	HPRPSB-1	26.12	5
40	HPRPSB-2	23.43	5
41	HPLPSB-2	49.22	3
42	HPLPSB-3	60.00	2
43	HPLPSB-4	26.83	5
44	HPLPSB-5	36.59	4
45	HPLPSB-6	69.27	1
46	HALPSB-1	26.82	5
47	HALPSB-2	17.07	6
48	FMLPSB-1	2.44	7
49	FMLPSB-2	42.00	3
50	FPTPSB-1	0.00	7
51	FPTPSB-2	36.12	4
52	FPTPSB-3	14.63	6
53	FPTPSB-4	26.83	5
54	FPRPSB-3	43.11	3
55	FPRPSB-4	13.90	6
56	YPTPSB-1	0.00	7
57	YPRPSB-1	22.93	5
58	YPLPSB-2	0.00	7
59	YPLPSB-3	21.34	5
60	YPLPSB-4	42.00	3
61	YALPSB-1	0.00	7

62	YALPSB-2	26.83	5
63	HPRB-1	43.43	3
64	HPRB-2	0.00	7
65	HPRB-4	0.00	7
66	HPRB-5	14.63	6
67	HPRB-6	26.83	5
68	HPRB-7	19.51	6
69	HPLB-1	21.95	5
70	HPLB-2	15.00	6
71	HPLB-3	45.00	3
72	HABB-1	65.00	1
73	HABB-2	50.00	3
74	HVZB-1	14.63	6
75	HVZB-2	7.32	7
76	HVZB-3	0.00	7
77	FPLB-2	11.11	6
78	FPLB-3	12.50	6
79	FPTB-1	10.00	7
80	FPTB-2	45.00	3
81	FMYB-1	12.20	6
82	YPRB-1	7.50	7
83	YPRB-2	5.00	7
84	YPLB-1	2.40	7
85	YPTB-1	4.80	7
86	YPTB-2	9.75	7
87	YABB-1	19.51	6
88	HPLBC-5	32.35	4
89	HPLBC-1	14.63	6
90	HPLBC-2	0.00	7
91	HPLBC-3	12.50	6
92	HABBC-1	53.66	2
93	HABBC-2	60.00	2

94	FPLBC-2	2.50	7
95	FPLBC-4	14.63	6
96	FPLBC-5	19.51	6
97	FPRBC-1	17.07	6
98	FPRBC-3	26.83	5
99	FMYBC-1	26.83	5
100	FMYBC-2	14.63	6
101	YPRBC-2	32.35	4
102	YPLBC-1	21.95	5
103	YPTBC-2	48.78	3
104	YPRBC-1	26.82	5
105	YPRBC-3	14.63	6
106	YMYBC-1	20.00	6
107	YMYBC-2	7.31	7
108	HPLPF-2	47.05	3
109	FPRPF-3	56.09	2
110	YABPF-2	12.19	6
111	FPTPF-1	19.51	6
112	FPTPF-2	56.10	2

* Ranks

0-9 %- 7

10-19 % - 6

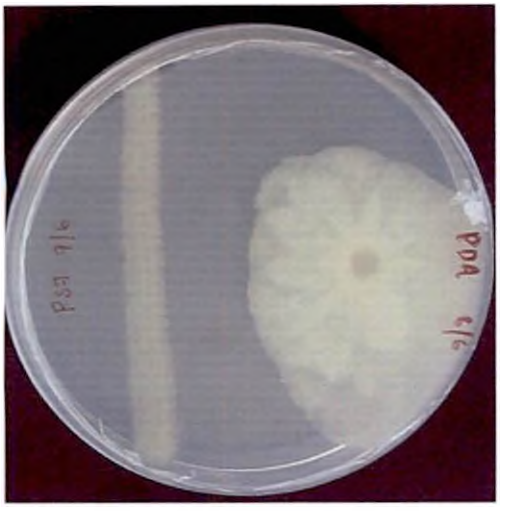
20-29 % - 5

30-39 % – 4

40-49 % - 3

50-59 % - 2

60-69 % - 1



HPLPSB-6

HABB-1

HPLPSB-3

Control

Plate 10. Dual culture of bacterial isolates and *P. capsici*

4.6.4. Screening of actinomycetes against *P. capsici*

The results of screening of actinomycetes against *P. capsici* are presented in the Table 19, Plate 11. Thirty two actinomycetes were evaluated for their antagonistic activity and were ranked based on their per cent inhibition.

Out of 32 isolates seven were ranked as first based on the per cent inhibition. Among these, isolate HVZACT-1 obtained from healthy garden gave 66.66 per cent inhibition. This was followed by HPLACT-2 (64.28), HVZACT-3 (62.50) from healthy gardens, FPTACT-2 (64.28), FPLACT-2 (62.50), FMYACT-2 (62.50) from foot rot affected gardens and YPRACT-1 (62.50) obtained from yellowing affected gardens.

Ten isolates, HVZACT-4 (57.50), HPRACT-2 (57.50), HPLACT-3 (57.50), HPLACT-4 (57.00) from healthy gardens, FPRACT-1 (57.00), FPTACT-1 (57.00), FMYACT-1 (55.00) from foot rot affected gardens andYPRACT-3 (57.14), YABACT-2 (55.00), YPTACT-3 (55.00) obtained from yellowing affected garden were scored the second rank.

Eight isolates, HPRACT-3 (47.60), HPLACT-1 (45.00), HABACT-2 (45.00), HPRACT-1 (42.50), HABACT2 (42.68) from healthy gardens and FPRACT-3 (49.00), FPRACT-1 (47.62), FPRACT2 (47.00) from foot rot affected gardens were scored the third rank.

Only one isolate YPRACT-2 obtained from yellowing affected garden which gave 37.00 per cent inhibition scored the fourth rank. Isolate YABACT-1 obtained from yellowing affected garden ranked fifth and gave 29.00 per cent inhibition. Two isolates ranked sixth and remaining three scored seventh rank.

4.6.5 Screening of fungal isolates against *P. capsici*

The efficiency of 63 fungal isolates were evaluated for antagonistic activity against *P. capsici* by dual culture. The results of the experiment are presented in Table 20 (Plate 12).

All the fungal isolates tested exhibited antagonistic activity against *P. capsici*. Among these, FPRF-3 obtained from foot rot affected garden recorded maximum inhibition of 100 per cent and this isolate scored first rank. Followed by isolates viz HPLF-3 (63.34%), HABF-1 (62.50%), HPRF-4 (62.50%) and HPLF-2 (62.50%) all obtained from healthy gardens.

Twenty seven isolates (14 from healthy gardens, 4 from foot rot affected gardens and 8 from yellowing affected garden) were placed in the second rank. The isolate YPRF-2 obtained from yellowing affected garden showed least per cent inhibition of 9.59 and it was ranked as 8. The isolates which scored first and second ranks were selected for testing the mechanism of antagonism.

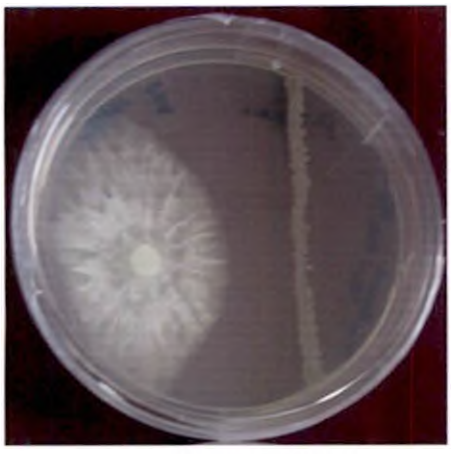
Table 19. Screening of actinomycetes against *P. capsici*

Sl. No	Isolates	Per cent inhibition	Rank
1	HPRACT-1	42.50	3
2	HPRACT-2	57.50	2
3	HPRACT-3	47.60	3
4	HPLACT-1	45.00	3
5	HPLACT-2	64.28	1
6	HPLACT-3	57.50	2
7	HPLACT-4	57.00	2
8	HVZACT-1	66.66	1
9	HVZACT-2	12.82	6
10	HVZACT-3	62.50	1
11	HVZACT-4	60.00	2
12	HABACT-1	45.00	3
13	HABACT-2	42.68	3
14	FPRACT-1	47.62	3
15	FPRACT-1	60.00	2
16	FPRACT-2	47.00	3
17	FPRACT-3	49.00	3
18	FPTACT-1	60.00	2
19	FPTACT-2	64.28	1
20	FPLACT-2	62.50	1
21	FMYACT-1	55.00	2

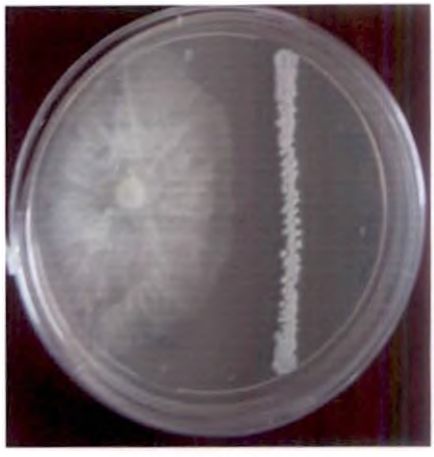
22	FMYACT-2	62.50	1
23	YPRACT-1	62.50	1
24	YPRACT-2	37.00	4
25	YPRACT-3	57.14	2
26	YPTACT-1	17.07	6
27	YPTACT-2	0.00	7
28	YPTACT-3	55.00	2
29	YABACT-1	29.00	5
30	YABACT-2	57.14	2
31	YABACT-3	0.00	7
32	YABACT-4	4.60	7

* Rank

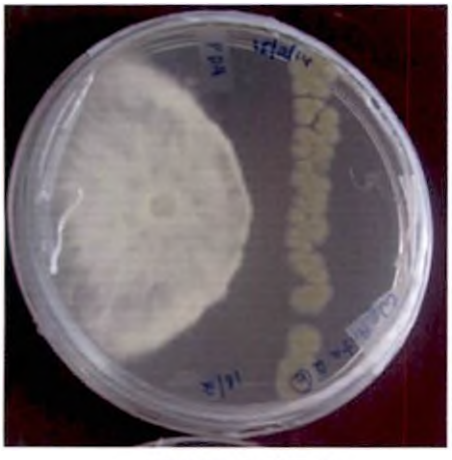
0-9 % - 7
10-19 % - 6
20-29 % - 5
30-39 % – 4
40-49 % - 3
50-59 % - 2
60-69 % - 1



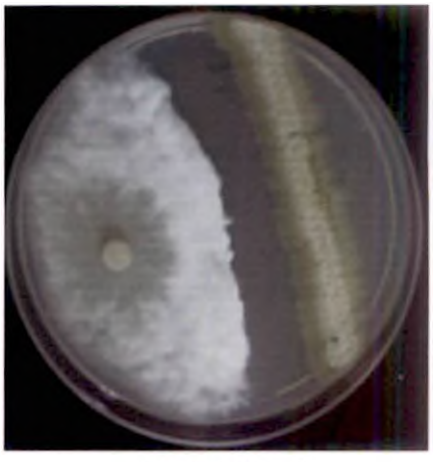
HVZACT-1



HPLACT-2

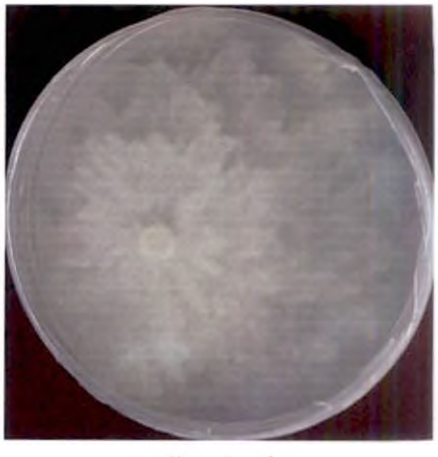


HVZACT-3



FPTACT-2

FPLACT-2



Control

Plate 11. Dual culture of actinomycetes and *P. capsici*

Table 20. Screening of fungal isolates against *P. capsici*

Sl.no	Isolates	Per cent inhibition	Rank
1	HPRF-1	56.25	3
2	HPRF-2	54.16	3
3	HPRF-3	55.41	3
4	HPRF-4	62.50	2
5	HPRF-5	55.41	3
6	HPRF-6	55.17	3
7	HPRF-7	55.84	3
8	HPLF-1	53.75	3
9	HPLF-2	62.50	2
10	HPLF-3	63.34	2
11	HPLF-4	54.16	3
12	HPLF-5	36.66	5
13	HPLF-6	54.59	3
14	HPLF-7	55.00	3
15	HABF-1	62.50	2
16	HABF-2	57.91	3
17	HABF-3	52.09	3
18	HABF-4	43.75	4
19	HABF-5	55.00	3
20	HVZF-2	57.50	3

21	HVZF-3	37.91	5
22	HVZF-4	48.75	4
23	HVZF-5	50.84	4
24	HVZF-6	51.25	3
25	FPRF-1	44.59	4
26	FPRF-2	48.75	4
27	FPRF-3	100	1
28	FPRF-4	48.75	4
29	FPRF-5	49.59	4
30	FPLF-2	40.84	4
31	FPLF-3	51.66	3
32	FPLF-4	50.84	4
33	FPLF-5	47.50	4
34	FPTF-1	40.84	4
35	FPTF-2	39.16	5
36	FPTF-3	36.66	5
37	FMYF-1	35.00	5
38	FMYF-2	48.75	4
39	FMYF-3	40.41	5
40	FMYF-4	58.34	3
41	FMYF-5	45.41	4
42	FMYF-6	56.66	3
43	YPRF-1	43.34	4
44	YPRF-2	9.59	8

45	YPRF-3	49.59	4
46	YPRF-4	49.16	4
47	YPRF-5	49.59	4
48	YPLF-1	42.91	4
49	YPLF-2	54.59	3
50	YPLF-3	54.59	3
51	YPLF-4	47.91	4
52	YPLF-5	58.34	3
53	YPTF-1	34.59	5
54	YPTF-2	55.84	3
55	YPTF-3	58.75	3
56	YPTF-4	39.16	5
57	YPTF-5	56.25	3
58	YPTF-6	44.59	4
59	YABF-1	56.66	3
60	YABF-2	57.91	3
61	YABF-3	52.09	3
62	YABF-4	44.16	4
63	YABF-5	45.00	4

* Ranks

0-9 % - 8

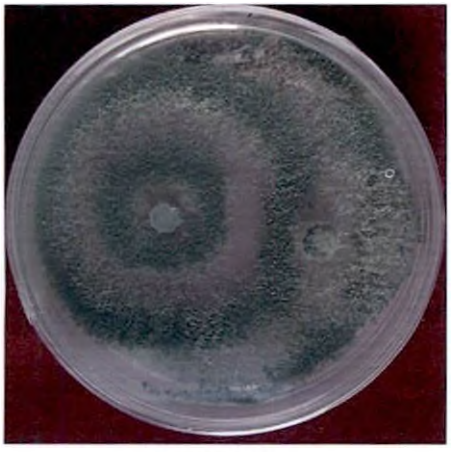
10-19 % - 7

20-29 % - 6

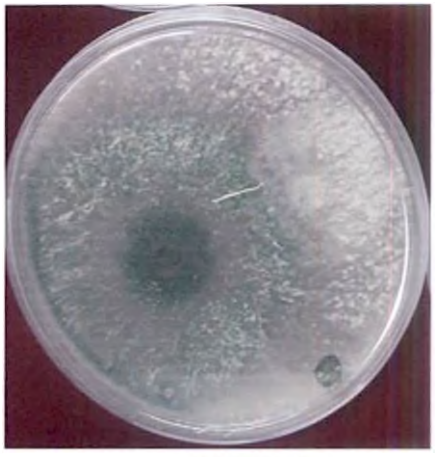
40-49 % - 4

50-59 % - 3

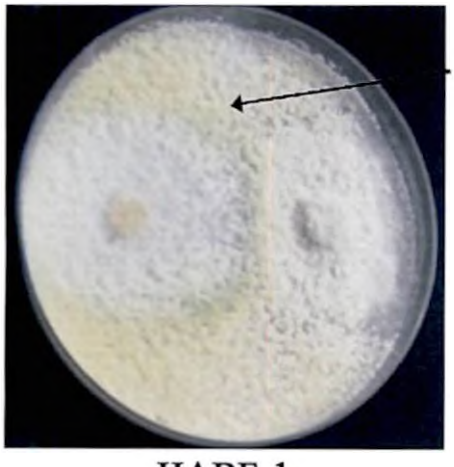
60-69 % - 2



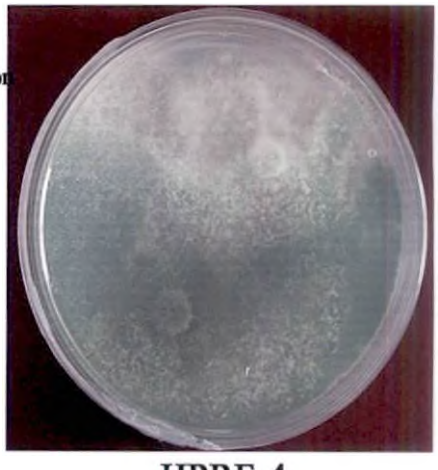
FPRF-3



HPLF-3



HABF-1



HPRF-4

HPLF-2

Control

Plate 12. Dual culture of Fungal isolates and *P. capsici*

30-39 % - 5 70-79 % - 1

4.6.6. Mechanism of antagonism

4.6.6.1. Effect of volatile metabolites from the antagonists against P. capsici

Selected isolates of antagonistic bacteria, fungi and actinomycetes which scored the first and second ranks were tested for the mechanism of antagonism such as HCN, ammonia and siderophore production.

4.6.6.1.1. HCN production

Seven selected bacterial antagonists proved to be HCN producers (Table 21). Among these, HPLPSB-6, HABB-1 & HABBC-1 obtained from healthy gardens were found to be good HCN producers. HPLPSB-3 and HABBC-1 from healthy gardens showed moderate HCN production whereas, FPTPF-2 and FPRPF-3 from foot rot affected gardens were found to be weak HCN producers (Plate 13A).

Among 17 actinomycete antagonists selected, eleven were confirmed to be HCN producers. HVZACT-3 and HVZACT-4 obtained from healthy gardens were classified under good HCN producers. HVZACT-1 from healthy garden and FPLACT-2, FPLACT-2, FPLACT-2&FPLACT-2from foot rot affected gardens were found to moderate HCN producers. HCN production was absent in case of antagonists HPLACT-3 and HPLACT-4 from healthy gardens, FMYACT-1 and FMYACT-2 from foot rot affected gardens and YPRACT-1 and YPRACT-3 from yellowing affected gardens.

Among the four fungal antagonists selected none were found to produce HCN.

4.6.6.1.2. Ammonia production

Among the seven bacterial antagonists tested for ammonia production, HPLPSB-6 and HABBC-2 obtained from healthy gardens were found to be good ammonia producers. HABB-1 from healthy garden and FPTPF-2 from foot rot affected garden were produces moderate of ammonia (Table 21, Plate 13 B).

All the selected actinomycetes were found to be positive for ammonia production. Among seventeen isolates tested, five isolates (HVZACT-1 & HPLACT-4 from healthy gardens; FPTACT-1, FPTACT-2 & FPRACT-1 from foot rot affected gardens) were found to be good ammonia producers. All other isolates were found to be moderate and weak producers of ammonia.

Four selected fungal antagonists were found to be weak producers of ammonia.

4.6.6.1.3. Effect of non-volatile metabolites on P. capsici

4.6.6.1.3.1. Siderophore production

Among seven bacterial antagonists tested, three (HPLPSB-6 & HPLPSB-3 from healthy gardens and FPTPF-2 from foot rot affected garden) were positive for siderophore production (Table 21, Plate 13 C).

Among the selected actinomycetes, five isolates (HVZACT-1, HPLACT-2, HVZACT-3, HPLACT-4 from healthy gardens and FPTACT-1 from foot rot affected garden) were confirmed as siderophore producers.

Fungal antagonists HPLF3 from healthy garden and FPRF3 from foot rot affected garden showed siderophore production.

Table 21. Production of metabolites by selected antagonists

Isolates	Volatile metabolites		Non-volatile metabolites
	HCN	Ammonia	Siderophore
Bacteria			
HPLPSB-6	+++	+++	+
HABB-1	+++	++	-
HPLPSB-3	++	+	+
HABBC-2	+++	+++	-
HABBC-1	++	+	-
FPTPF-2	+	++	+
FPRPF-3	+	+	-
Actinomycetes			
HVZACT-1	++	+++	+
HPLACT-2	+	++	+
FPTACT-2	+	+++	-
HVZACT-3	+++	+	+
FPLACT-2	++	+	-
FMYACT-2	-	++	+
YPRACT-1	-	+	-
HVZACT-4	+++	++	-
FPRACT-1	++	+++	-

FPTACT-1	+	+++	+
YABACT-2	+	+	-
HPRACT-2	++	+	-
HPLACT-3	-	++	-
HPLACT-4	-	+++	+
YPRACT-3	-	+	-
FMYACT-1	-	+	-
YPTACT-3	+	+	-
Fungi			
FPRF-3	-	+	+
HPLF-3	-	+	+
HPRF-4	-	-	-
HPLF-2	-	+	+

+++ Good ++ Moderate + Weak

4.6.6.1.3.2. Effect of culture filtrate on radial growth of *P. capsici*

Effect of culture filtrate on radial growth of selected best antagonists were observed to detect the production of non volatile compounds. All the selected antagonists were found to inhibit the mycelial growth of *P. capsici* (Table 22)

All the seven bacterial antagonists (HPLPSB-6, from healthy garden; HABB-1, from healthy garden; HPLPSB-3, from healthy garden; HABBC-2, from healthy garden; FPTPF-2, from foot rot affected garden) inhibited the full growth of *P. capsici* and per cent inhibition of 100 per cent was recorded.

Among the actinomycetes, ten isolates viz. HVZACT-1, HPLACT-2, HVZACT-3, HPLACT-3, & HPLACT-4, FPLACT2, FPTACT-1, FPTACT-2 & FMYACT-2 and YPRACT-1 recorded 100 per cent inhibition on the mycelial growth of *P. capsici.*

Among the four fungal antagonists selected FPRF-3 (from foot rot affected garden) and HPLF-3 (from the healthy garden) recorded 100 per cent inhibition on mycelial growth of *P. capsici.*HPRF-3 and HPLF-2 obtained from healthy pepper gardens showed inhibition with 83.30 and 81.48 respectively.

Table 22.Effect of culture filtrate on radial growth of *P. capsici*

Isolates	Per cent growth inhibition of *P. capsici*
Bacteria	
HPLPSB-6	100.00
HABB-1	100.00
HPLPSB-3	100.00
HABBC-2	100.00
FPTPF-2	100.00
FPRPF-3	100.00
HABBC-1	100.00
Actinomycetes	
HVZACT-1	100.00
HPLACT-2	100.00
FPTACT-2	100.00
HVZACT-3	100.00
FPLACT-2	100.00
FMYACT-2	77.77
YPRACT-1	100.00
HVZACT-4	83.30

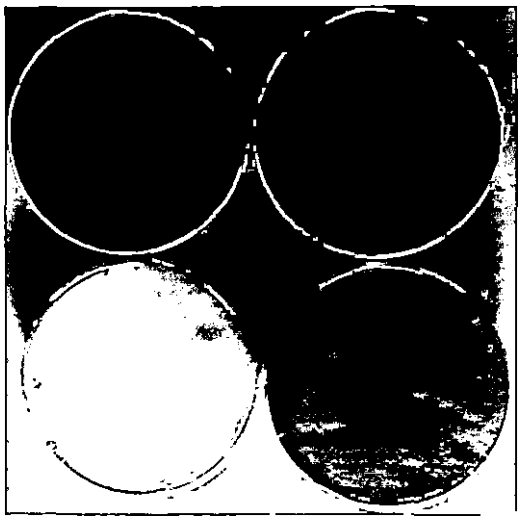
FPRACT-1	81.30
FPTACT-1	100.00
YABACT-2	75.30
HPRACT-2	74.07
HPLACT-3	100.00
HPLACT-4	100.00
YPRACT-3	80.25
FMYACT-1	75.30
YPTACT-3	80.00
Fungi	
FPRF-3	100.00
HPLF-3	100.00
HPRF-4	83.30
HPLF-2	81.48

4.6.6.3. Microscopic examination for hyphal interaction

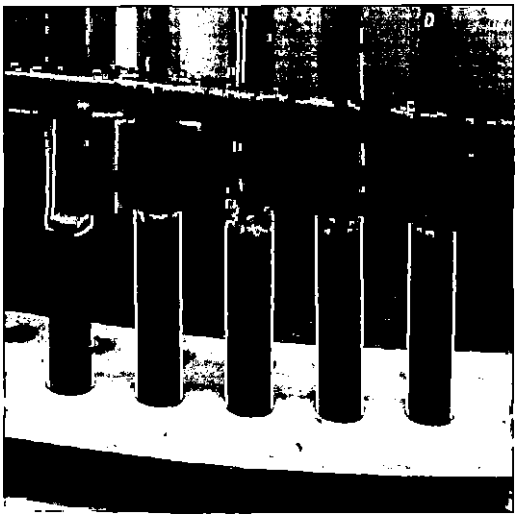
Four selected fungal isolates were observed for mycoparasitism under microscope and the results are presented in Table 16, Plate 13 D.

Table 23. Hyphal interactions by the fungal antagonists

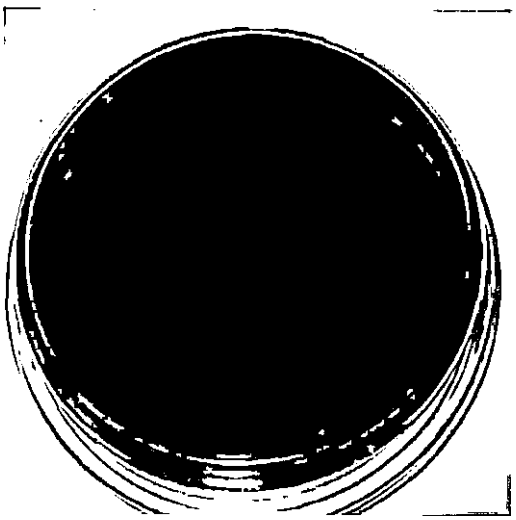
Selected fungal isolates	Hyphal interactions
FPRF-3	Hyphae intermingling and coiling
HPLF-3	Hyphae intermingling and coiling
HPRF-4	Hyphae intermingling and coiling
HPLF-2	Hyphae intermingling and coiling



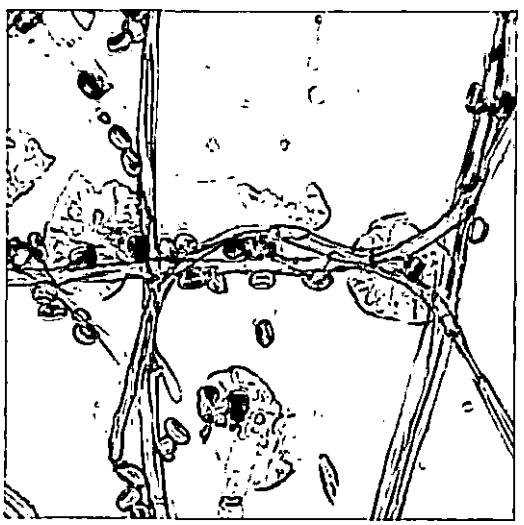
A. HCN Production



B. Ammonia Production



C. Siderophore Production by HPLPSB-6



D. Mycoparasitism of *P. capsici* by fungal antagonist FPRF-3

Plate 13. Mechanism of antagonism of PGPM on *P. capsici*

4.7. EVALUATION OF SELECTED ISOLATES FOR ANTAGONISTIC ACTIVITY IN BLACK PEPPER CUTTINGS UNDER *IN PLANTA* CONDITIONS

Three most efficient PGPM selected from *in planta* screening for growth promotion and three most promising antagonists including bacteria, an actinomycete and a fungus were evaluated for their efficiency in controlling foot rot disease in black pepper cuttings, as detailed in section 3.2.6.1 under Materials and Methods.

4.6.7.1. Biometric observations

4.6.7.1.1. Disease incidence

Challenge inoculation with *P. capsici* was carried out one month after the first application of bioinoculants. Per cent disease incidence of foot rot disease was recorded for each treatment separately one month after challenge inoculation. The results obtained are presented in Table 24, Plate 15-16.

Treatment T_6 (FPRF-3, from foot rot affected pepper garden) recorded the minimum incidence of disease (6.23 per cent) and this was found to be statistically superior to all other treatments except T_5 (HVZACT-1, from healthy pepper garden) with a disease incidence of 13.20 per cent. Maximum disease incidence (93.77 per cent) was noticed in T_9 (control with pathogen alone).

4.6.7.1.2. Disease severity

Based on the score chart given in Plate 14, disease severity of foot rot disease was calculated for each treatment after one month of challenge inoculum. The results obtained are shown in Table 24.

Plants treated with T_6 (FPRF-3, from foot rot affected pepper garden) recorded the least disease severity (4.00 per cent) and this was found to be

statistically superior to all treatments except T_5 (HVZACT-1, from healthy garden) with a disease severity of 8.00 per cent. The per cent disease severity was maximum in T_9 (control with pathogen alone) with disease severity of 90.00 per cent. Treatments T7 (*Pseudomonas fluorscens* [KAU]) with 84.00 per cent and T_4 (HPLPSB-6, obtained from healthy pepper garden) with 65.33 per cent respectively, were on par with T_9.

4.6.7.1.3. Length of vines

Effect of bioinoculants on the length of vine are presented in Table 25. Intial length of pepper vines in all treatments was almost uniform. After the application of bioinoculants an increase in length of vines was noticed in all treatments.

After 30 days of bioinoculants application, treatment T_6 (FPRF3, from foot rot affected garden) recorded maximum length of vine (37.13cm) and this was found to be statistically superior to all other treatments. This was followed by T_8 (*Trichoderma viride*) and T_5 (HVZACT-1) which recorded vine length of 30.17cm and 29.85cm respectively. Minimum length of 20.03cm was observed in T_9 (control with pathogen alone).

After 60 days of bioinoculant application, treatment T_6 (FPRF3) recorded maximum length of 46.40cm and found to be significantly superior to all other treatments. This was followed by T_5 (HVZACT1) and T_8 (*Trichoderma viride*) which recorded vine length of 38.03cm and 37.72cm respectively. Minimum length of 20.03cm was observed in T_9 (Control with pathogen alone).

Table 24. Effect of PGPM and antagonists on PDI and PDS

Treatments		Per cent disease incidence (PDI)	Per cent disease severity (PDS)
T_1	YPTN-3: *Paenibacillus* sp.	73.60(8.55^{ab})	53.33(7.20^{c})*
T_2	HPLPSB-3: *Acinetobacter grimontii*	67.33(8.20^{ab})	46.17(6.67^{c})
T_3	HPLBC-6: *Providencia* sp.	66.67(8.15^{ab})	58.33(7.66b^{c})
T_4	HPLPSB-6: *Paenibacillus polymyxa*	71.53(8.45^{ab})	65.33(8.10^{abc})
T_5	HVZACT-1: *Streptomyces termitum*	13.20(3.67^{c})	8.00(2.08^{d})
T_6	FPRF-3: *Trichoderma viride*	6.23(2.52^{c})	4.00(2.03^{d})
T_7	*Pseudomonas fluorescens* P1 (KAU)	87.50(9.36^{a})	84.00(9.17^{ab})
T_8	*Trichoderma viride*	66.00(8.07^{ab})	56.67(7.53^{bc})
T_9	Control with pathogen alone	93.77(9.71^{a})	90.00(9.50^{a})
T_{10}	Absolute control	54.17(7.38^{b})	57.33(9.60^{bc})

*Square root transformed values are given in paranthesis

A. Score 0: no infection

B. Score 1: < 10% infection

C. Score 2: > 10-25% infection

D. Score 3: > 25-50% infection

E. Score 4: > 50-75% infection

F. Score 5: > 75% or defoliated

Plate. 14. Score chart for severity of foot rot disease

T_1 (*Paenibacillus* sp.)

T_2 (*Providencia* sp.)

T_3 (*Acinetobacter grimontii*)

T_4 (*Paenibacillus polymyxa*)

T_5 (*Streptomyces termitum*)

T_6 (*Trichoderma viride*)

Plate 15. Effect of PGPM on incidence of foot rot disease (10 days after symptom appearance)

T_7 (*Pseudomonas fluorescens* KAU)

T_8 (*Trichoderma viride* -KAU)

T_9 (Control with pathogen alone)

T_{10} (Absolute control)

Plate 16: Effect of PGPM on incidence of foot rot disease (10 days after symptom appearance)

Table 25. Effect of PGPM and antagonists on length of vine

Treatments		Length of vine		
		Intial length	30 days after bioinoculants application	60 days after bioinoculants application
T_1	YPTN-3: *Paenibacillus* sp.	16.66	20.26^{d}	29.58^{cd}
T_2	HPLPSB-3: *Acinetobacter grimontii*	16.96	21.27^{d}	25.44^{de}
T_3	HPLBC-6: *Providencia* sp.	16.17	25.79^{c}	33.20^{c}
T_4	HPLPSB-6: *Paenibacillus polymyxa*	16.61	25.97^{c}	32.35^{c}
T_5	HVZACT-1: *Streptomyces termitum*	16.25	29.85^{b}	38.03^{b}
T_6	FPRF-3: *Trichoderma viride*	16.11	37.13^{a}	46.40^{a}
T_7	*Pseudomonas fluorescens* P1 (KAU)	16.23	20.19^{d}	23.35^{e}
T_8	*Trichoderma viride*	16.25	30.17^{b}	37.72^{b}
T_9	Control with pathogen alone	16.39	20.03^{d}	24.21^{e}
T_{10}	Absolute control	16.35	22.05^{d}	25.20^{de}

4.6.7.1.4. Number of leaves

Effect of PGPM and antagonists on the number of leaves were observed after 30 and 60 days of bioinoculant application. Intial number of leaves was the same in all the treatments, as seedlings at two leaf stage were choosen for the experiment. The observations are presented in Table 26.

Thirty days after bioinoculant application T_6 (FPRF-3) was found to be statistically superior with number of leaves 3.67. All other treatments were found to be statistically on par with each other. Minimum number of leaves (2.32) was observed in T_2 (HPLPSB-3).

Similar trend was followed after 60 days of bioinoculant application. Treatment T_6 (FPRF3) was found to be statistically superior with number of leaves (5.84) and which was on par with T_8 (*Trichoderma viride*) and T_1 (YPTN-3) having 4.64 and 4.63 respectively. Minimum number of leaves (3.43) was observed in treatment T_{10} (absolute control).

Table 26. Effect of PGPM and antagonists on number of leaves

Treatments		Number of leaves per vine		
		Initial *	30 days after bioinoculant application	60 days after bioinoculant application
T_1	YPTN-3: *Paenibacillus* sp.	2	2.75^b	4.63^{ab}
T_2	HPLPSB-3: *Acinetobacter grimontii*	2	2.32^b	3.67^b
T_3	HPLBC-6: *Providencia* sp.	2	2.50^b	4.17^b
T_4	HPLPSB-6: *Paenibacillus polymyxa*	2	2.38^b	3.67^b
T_5	HVZACT-1: *Streptomyces termitum*	2	2.57^b	4.00^b
T_6	FPRF-3: *Trichoderma viride*	2	3.67^a	5.84^a
T_7	*Pseudomonas fluorescens* P1 (KAU)	2	2.83^b	3.67^b
T_8	*Trichoderma viride*	2	2.73^b	4.64^{ab}
T_9	Control with pathogen alone	2	2.75^b	4.00^b
T_{10}	Absolute control	2	2.75^b	3.43^b

*Significantly not different

4.6.7.1.5. Number of roots

Seedlings were uprooted and number of roots were counted after 60 days of bioinoculants application. Data obtained are presented in Table 27. Maximum number of roots (7.53) were observed in treatments T_8 (*Trichoderma viride*). This was found to be on par with all treatments except T_7 (*Pseudomonas fluorescens* [KAU]).

4.6.7.1.6. Length of roots

Maximum root length (16.40cm) was noticed in T_4 (HPLPSB-6, from healthy garden) and this was on par with T_1 (13.76cm), T_3 (14.91cm) and T_6 (14.00cm). All these treatments were statistically superior to T_2 (10.54cm), T_5 (12.49cm), T_7 (11.95cm), T_8 (12.35cm), T_9 (9.38cm) and T_{10} (6.98cm). The results are given in Table 27.

4.6.7.1.7. Fresh weight of roots

Fresh weight of roots was highest (0.88g) in T_8 (*Trichoderma viride*). This was followed by T_6 (FPRF-3). These were on par with T_1 (YPTN-3), T_2 (HPLPSB-3, from healthy garden), T_3 (HPLBC-6), T_4 (HPLPSB-6) and T_5 (HVZACT-1) with 0.71g, 0.49g, 0.76g, 0.64g and 0.46g respectively. All these treatments were statistically superior to T_7 (0.29g), T9 (0.29g) and T_{10} (0.21g). the observations are presented in Table 27.

Table 27. Effect of PGPM and antagonists on root parameters

Treatments		Root parameters (per vine)		
		Number	Length (cm)	Fresh weight (g)
T_1	YPTN-3: *Paenibacillus* sp.	7.50[a]	13.76[abc]	0.71 [abc]
T_2	HPLPSB-3: *Acinetobacter grimontii*	5.21 [ab]	10.54[cd]	0.49 [abc]
T_3	HPLBC-6: *Providencia* sp.	7.29[a]	14.91 [ab]	0.76 [ab]
T_4	HPLPSB-6: *Paenibacillus polymyxa*	4.00[ab]	16.40 [a]	0.64 [abc]
T_5	HVZACT-1: *Streptomyces termitum*	6.81[a]	12.49[bcd]	0.46 [abc]
T_6	FPRF-3: *Trichoderma viride*	7.42 [a]	14.00 [ab]	0.83 [a]
T_7	*Pseudomonas fluorescens* P1 (KAU)	2.28[b]	11.95 [bcd]	0.29 [bc]
T_8	*Trichoderma viride*	7.53 [a]	12.35 [bcd]	0.88 [a]
T_9	Control with pathogen alone	5.86 [ab]	9.38[d]	0.29 [bc]
T_{10}	Absolute control	4.63 [ab]	6.98[e]	0.21 [c]

4.7. IDENTIFICATION OF SELECTED PGPM AND ANTAGONISTS

Six PGPM and three antagonists selected from *in vitro* and *in planta* screening were identified based on their colony and microscopic characters (Plate 17 – 18). In case of bacteria and actinomycetes 16S rDNA sequence analysis was carried out for identification.

4.7.1. Identification of selected bacterial isolates

4.7.1.1. Cultural and morphological characters

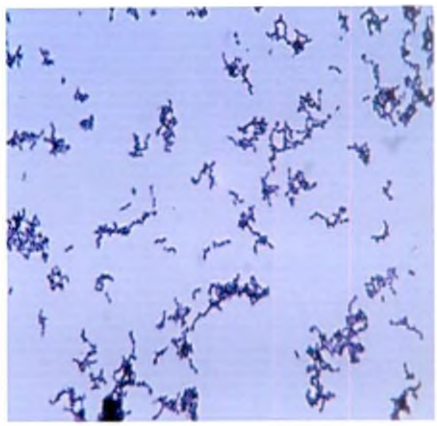
Cultural and morphological characters of the selected bacterial isolates were studied in detail on nutrient agar medium and the observations are given in Table 28.

4.7.1.2. 16S rDNA sequence analysis

After observing the cultural and morphological characters of the bacterial isolates, they were identified by 16S rDNA sequence analysis. Homology search of nucleotide sequences obtained from the isolates with other reported sequences are presented in Table 29. The isolates YPTN-3 and HVKN-6 showed maximum homology with *Paenibacillus* sp., HPLPSB3 with *Acinetobacter grimontii*,HPLBC-6 with *Providencia* sp., HABBC3 with *Providencia* sp. and HPLPSB6 with *Paenibacillus polymyxa.* The test isolates were identified as the culturable accession sharing maximum homology with query sequence (Plates 19-23).

Table 28. Colony morphology of selected bacterial isolates

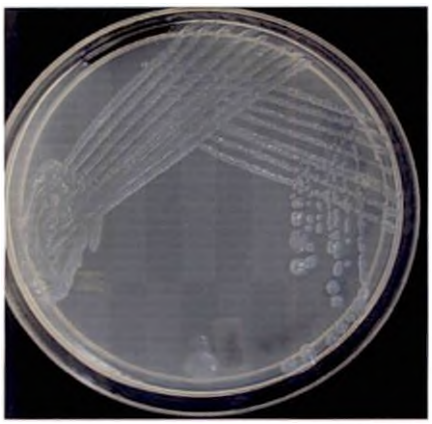
Isolates	Colony morphology					Gram reaction	Cell morphology
	Shape	Colour	Surface	Margin	Elevation		
YPTN-3	Circular	Colourless	Viscous	Entire	Pulvinate	+ve	Rods
HVKN-6	Circular	Colourless	Slimy	Entire	Convex	+ve	Rods
HPLPSB-3	Circular	Cream	Rough	Entire	Flat	-ve	Rods
HPLBC-6	Circular	Cream	Smooth	Entire	Flat	-ve	Rods
HABBC-3	Circular	White	Smooth	Entire	Flat	-ve	Rods
HPLPSB-6	Circular	Cream	Slimy	Entire	Convex	+ve	Rods

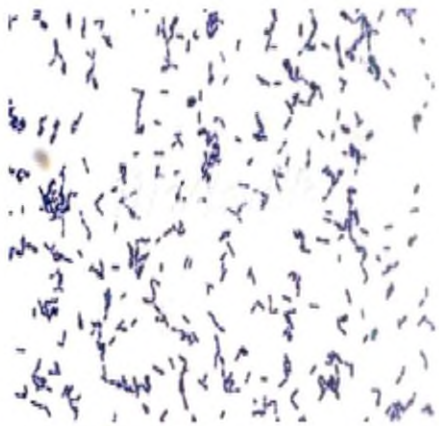


YPTN-3 (*Paenibacillus* sp.)

Colonies on Jensen's agar

Microscopic view (1000X magnification)

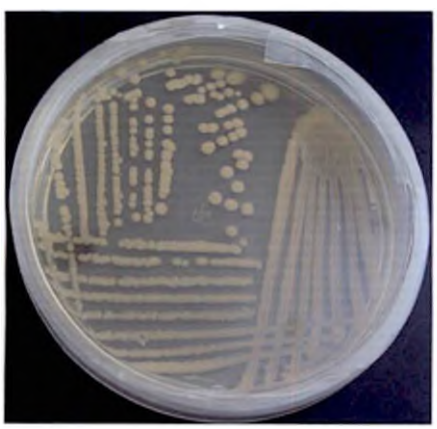


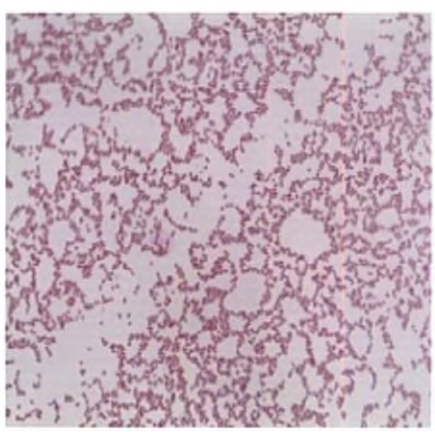


HVKN-6 (*Paenibacillus* sp.)

Colonies on Jensen's agar

Microscopic view (1000X magnification)



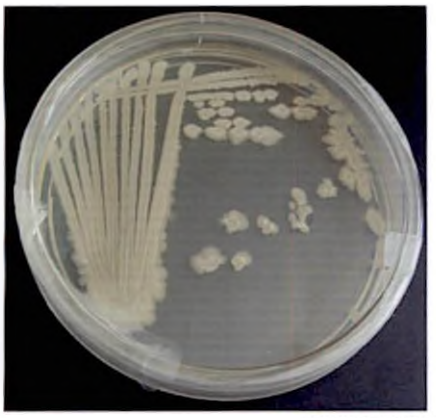


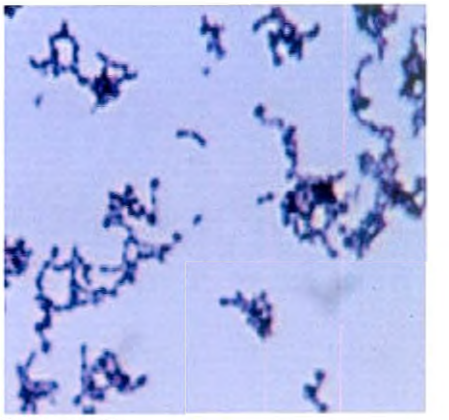
HPLPSB-3 (*Acinetobacter grimontii*)

Colonies on nutrient agar medium

Microscopic view (1000X magnification)

Plate 17. Colony morphology and microscopy of PGPM (bacteria and actinomycetes)

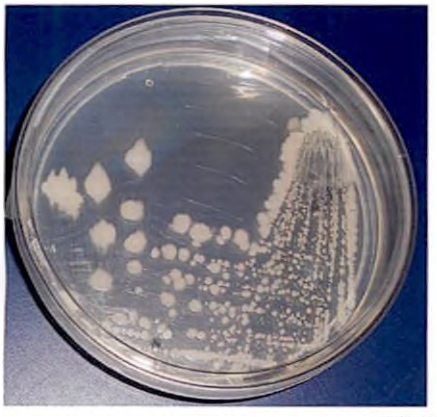




HPLPSB-6 (*Paenibacillus polymyxa*)

Colonies on nutrient agar medium

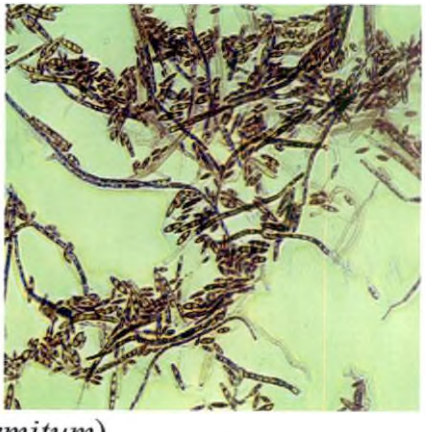
Microscopic view
(1000X magnification)



HPLBC-6 (*Providencia* sp.)

Colonies on nutrient agar medium

Microscopic view
(1000X magnification)



HVZACT-1 (*Streptomyces termitum*)

Colonies on Kenknight's agar medium

Microscopic view
(1000X magnification)

Plate 18. Colony morphology and microscopy of PGPM (bacteria and actinomycetes)

Table 29. 16S rDNA sequence analysis of selected bacterial isolates

Isolates	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e value
	Accession no.	Name				
YPTN-3	JX133591.1	Uncultured bacterium	2396	96	97	0.0
	HQ728394.1	Bacterium 47(2011)	2381	96	97	0.0
	JX133644.1	Uncultured bacterium	2377	96	97	0.0
	EU592044.1	*Paenibacillus* sp.	2370	94	97	0.0
	AB089251.1	*Paenibacillus* sp.	2357	93	97	0.0
HVKN-6	EU692044.1	*Paenibacillus* sp.	2207	93	97	0.0
	JX133591.1	Uncultured bacterium	2191	93	97	0.0
	HQ728394.1	Bacterium 47(2011)	2182	93	97	0.0
	JX133644.1	Uncultured bacterium	2180	93	97	0.0
	KJ880866.1	Bacterium SB-6	2178	94	98	0.0
HPLPSB-3	AM410706.2	*Acinetobacter grimontii*	2259	98	99	0.0
	KF663060.1	*Acinetobacter* sp.	2255	98	98	0.0
	KF798784.1	Uncultured bacterium clone	2255	98	98	0.0
	AB690765.1	Uncultured gamma proteobacterium	2255	98	98	0.0
	AB860303.1	*Acinetobacter junii* gene	2255	98	98	0.0
HPLBC-6	KC755066.1	Uncultured *Providencia* sp.	2311	98	99	0.0
	NR104913.1	*Providencia sneebia*	2255	98	98	0.0
	KF471514.1	*Providencia vermicola*	2250	98	98	0.0
	KF471512.1	*Providencia vermicola*	2250	98	98	0.0
	HM625779.1	*Providencia* sp.	2246	98	98	0.0
HABBC-3	KC755066.1	*Providencia* sp.	2318	98	99	0.0
	NR104913.1	*Providencia sneebia*	2263	98	98	0.0
	KF471514.1	*Providencia vermicola*	2257	98	98	0.0
	KF471512.1	*Providencia vermicola*	2257	98	98	0.0
	KF193115.1	Bacterium endosymbiont of *Onthophagus Taurus*	2252	98	98	0.0
HPLPSB-6	AY359621.1	*Paenibacillus polymyxa*	2052	97	97	0.0
	HQ891961.1	*Paenibacillus* sp.	2045	97	97	0.0
	GU332610.1	*Paenibacillus polymyxa*	2045	97	97	0.0

	EF690401.1	*Paenibacillus polymyxa*	2041	97	97	0.0
	JX266302.1	*Paenibacillus* sp.	2034	97	97	0.0

4.7.2. Identification of selected actinomycete

4.7.2.1. Cultural and morphological characters

Cultural and morphological characters of the selected actinomycete was studied in detail on Kenknight's agar medium and based on that it was identified as *Streptomyces* sp. Table 30, Plate 18.

4.7.2.2. 16S rDNA sequence analysis

Species level identification of the actinomycete HVZACT-2 was carried out by 16S rDNA sequence analysis Table 31.Homology search of nucleotide sequences showed maximum homology with *Streptomyces termitum.* Plate 24.

TAGATCCCCACTTAGCGCTCTATCTTCCCAAATCATACTACCACCACCTTCGACGGC-
TAGCTCCCTTGCGGGTTACCCCACCGGCTTCGGGTGTTGTAAACTCTCGTGGTGTGACGGGCGGTGTGTACAAGACCCGGGAACGT
ATTCACCGCGGCATGCTGATCCGCGATTACTAGCAATTCCGACTTCATGCAGGCGAGTTGCAGCCTGCAATCCGAACTGAGACCGAC
TTTGGTAGGATTGGCTCCGCCTCGCGGCTTCGCTTCCCGTTGTATCGGCCATTGTAGTACGTGTGTAGCCCAGGTCATAAGGGGCATG
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AAGGTTGCGTCGTTGCGGGACTAACCCAACATCTCAGACACGAGCGAGACAAACATGCACACCTGTCTCCTCGCCCCGAAGGGAA
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TGCGGGTCCCCGTCAATTCCTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAATGCTAATGTGTTAACTTCGGCACCAAGGG
TATCGAAACCCCTAACACCTAGCATTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCT
CAGCGTCAGTTACAGCCCAGAAAGTCGCCTTCGCCACTGGTGTTCCTCAACATCTCTACGCATTTCACCGCTACACGTGGAATTCCA
CTTTCCTCTTCTGCACTCAAGCCAACAATTTCCAGTGCGAACCGGGGTTGAGCCCCACCTTAAACACAGACTTAATTAACCGCCTGC
GCGCGCTTTACGCCAATATTCCGGACACGCTTGCCCCCTACTATTACCGAGGCTGCTGACCGAATTAGCCGGGGCTTTCTTCTCAGGT
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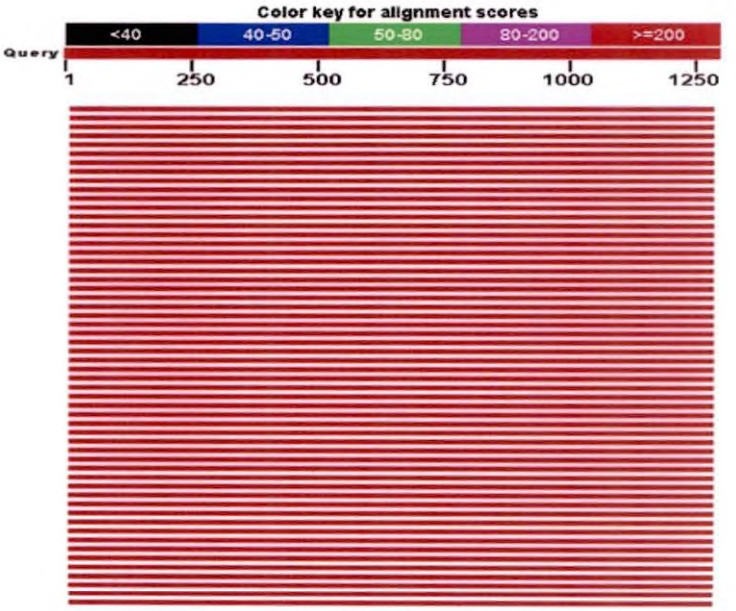
GGCCGATCACCCTCTCAGGTCGGCTACGCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCAGGTCCATCT
GTAAGTGACAGATTGCTCCGTCTTTCCCGAATCGGTCATGCGACCAATTCGCGTATCCGGTATTAGCATTCGTTTCCGAATGTTATCC
CGGTCTTACAGGCAGGTTACCTACGTGTTACTCACCCGTCCGCCGCTAACCTTGTCCCGAAGGACAAGATCCGCTCGACTGTAATGT
GGAGGTCATATTTGGTTATTGGAG

A. Sequence of 16S rDNA amplicon



B. Blastn ouput

NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e value
Accession no.	Name				
JX133591.1	Uncultured bacterium	2396	96	97	0.0
HQ728394.1	Bacterium 47(2011)	2381	96	97	0.0
JX133644.1	Uncultured bacterium	2377	96	97	0.0
EU592044.1	*Paenibacillus* sp.	2370	94	97	0.0
AB089251.1	*Paenibacillus* sp.	2357	93	97	0.0

C. Sequences showing homology

Plate. 19. Sequence analysis of isolate YPTN-3

TAGCCGGTGCCGCGCTTACACTTATCTTAGCGGCGGACGGGTGAGTAATGCTTAGGAATCTGCCTATTAGTGGGGGACAACATTCCG
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GCTAATAGATGAGCCTAAGTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATG
ATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGGAACCCTGAT
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ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTTTCCTTACTTGCCAGCATTTCGGATGGGAACTTTAAGGATACTGCCAGTGAC
AAACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAG
GGTTGCTACACAGCGATGTGATGCTAATCTCAAAAAGCCGATCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGA
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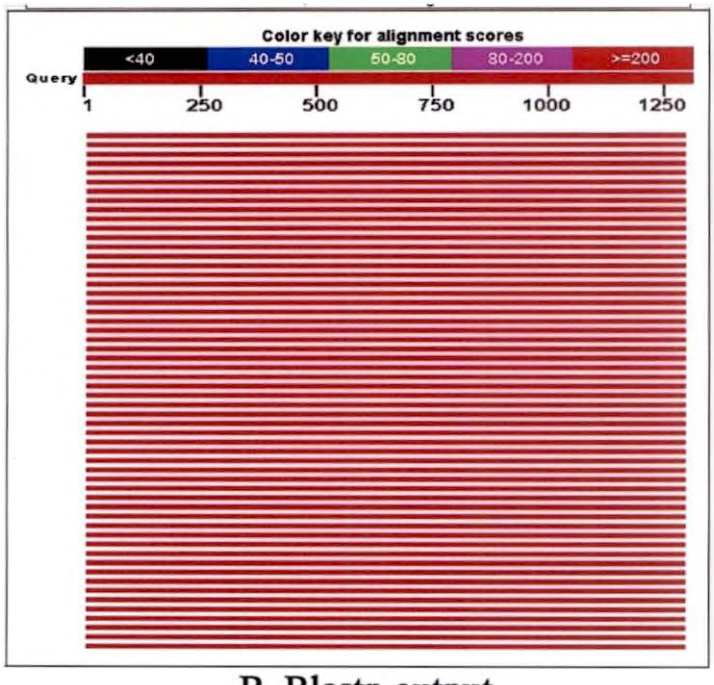
A. Sequence of 16S rDNA amplicon



B. Blastn output

NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e value
Accession no.	Name				
EU692044.1	*Paenibacillus* sp.	2207	93	97	0.0
JX133591.1	Uncultured bacterium	2191	93	97	0.0
HQ728394.1	Bacterium 47(2011)	2182	93	97	0.0
JX133644.1	Uncultured bacterium	2180	93	97	0.0
KJ880866.1	Bacterium SB-6	2178	94	98	0.0

C. Sequence showing homology

Plate 20. Sequence analysis of isolate HVKN-6

AAAGCATCGCAGCTTCTGATCTACGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTG-
CAGACTCCAATCCGGACTACGACGTACTTTATGAGTTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATACGCCATTGTAGCACGT
GTGTAGCCCTACTCGTAAGGGCCATGATGACTGAAAGTCATCCCCACCGTCCTACAGTTTATCACTGGCAGTCTCCTTGGAGTTCCC
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CGACTGAAAGTACTTTACAACCCTAGGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCATTGTGCAATATTCCCC
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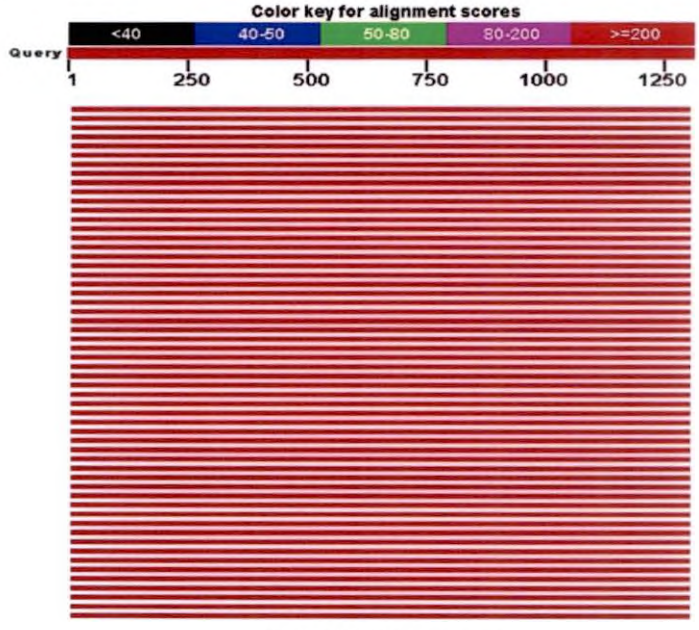
GTGAGCCTTTACCTCACCTACTAGCTAATCCCATATGGGTTCATCCGATAGCGCAAGGACCTAAGTTCCCCTGCTTTGCTCCAAAGAG
ATTATGCGGTATTAGCCACCGTTTCCAGTGGTTATCCCCCTCTATCGGGCAGATCCCCATACATTACTCACCCGTCCGCCGCTCGTCA
GCGAGAAGCTAGCTGTGCCAGCTCGAA

A. Sequence of 16S rDNA amplicon



B. Blastn ouput

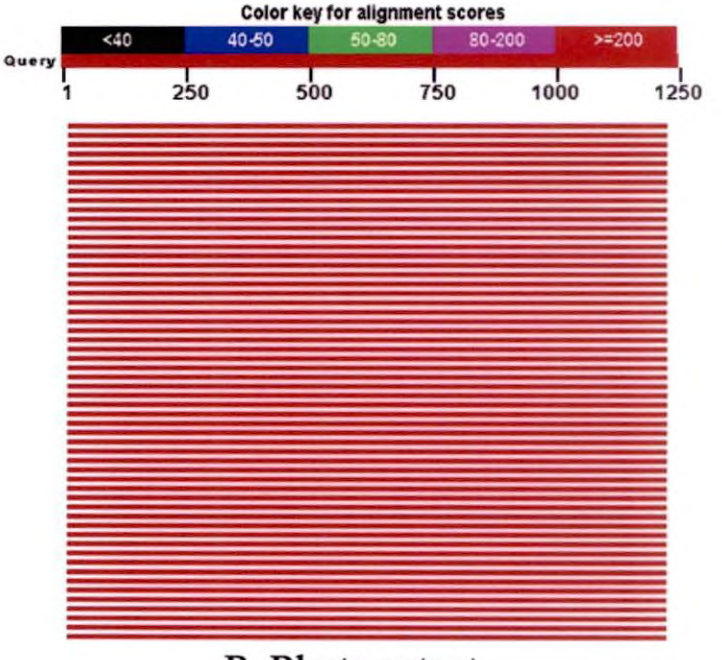
Description	Max score	Total score	Querv cover	Identity	Accession
Uncultured *Providencia* sp.	2318	2318	98%	99%	KC755066.1
Providencia sneebia	2263	2263	98%	98%	NR_104913.1
Providencia vermicola	2257	2257	98%	98%	KF471514.1
Providencia vermicola	2257	2257	98%	98%	KF471512.1
Bacterium endosymbiont of *Onthophagus Taurus*	2252	2252	98%	98%	KF193115.1

C. Sequence showing homology

Plate 21. Sequence analysis of isolate HPLBC-6

GTACGGCTGGCACAGCTAGCTTCTCGCTGACGAGCGGCGGACGGGTGAGTAATG-
TAGGGGATCTGCCCGATAGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAATCTCTTTGGAGCAAAGCAGGGGAACTT
AGGTCCTTGCGCTATCGGATGAACCCATATGGGATTAGCTAGTAGGTGAGGTAAAGGCTCACCTAGGCGACGATCCCTAGCTGGTCT
GAGAGGATGATCAGCAACACTGGGACTGAGACACGGCCCAGATCCTACGGGAAGCAGCAGTGGGGAATATTGCACAATGGGCGCA
AGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGACCCTAGGGTTGTAAAGTACTTTCAGTCGGGAGGAAGGCGTTGGCGCTAATA
TCGTCAACGATTGACGTTACCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTA
ATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGATTAAGTTAGATGTGAAATCCCCGGGCTTAACCTGGGAATGGCATCTAA
GACTGGTCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAATACCGGTGGC
GAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCT
GTAAACGATGTCGATTTGGAGGTTGTTCCCTAGAGGAGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCC
GCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGACCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
CCTACTCTTGACATCCAGAGAACTTAGAAGAGATGCTTTGGTGCCTTCGGGAACTCTGAAACAGGAGCTGCATGGCTGTCGTCAGC
TCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGA
GACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATG
GCGTATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGAACTCATAAAGTACGTCGTAGTCCGGATTGGAGTCTGCAACTCGACT
CCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAAGCTACGAGGCCGCC

A. Sequence of 16S rDNA amplicon



B. Blastn output

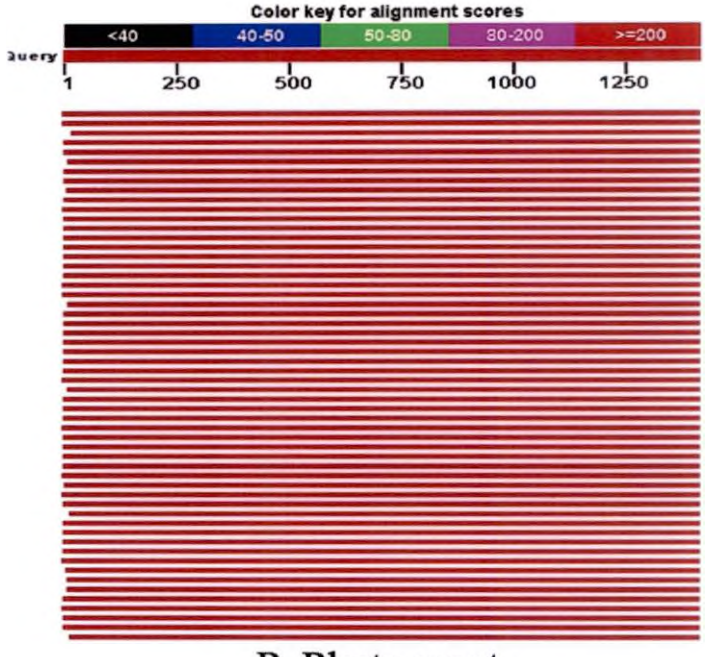
Isolates	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e value
	Accession no.	Name				
HABBC-3	KC755066.1	*Providencia* sp.	2318	98	99	0.0
	NR104913.1	*Providencia sneebia*	2263	98	98	0.0
	KF471514.1	*Providencia vermicola*	2257	98	98	0.0
	KF471512.1	*Providencia vermicola*	2257	98	98	0.0
	KF193115.1	Bacterium endosymbiont of *Onthophagus Taurus*	2252	98	98	0.0

C. Sequence showing homology

Plate 22. Sequence analysis of isolate HABBC-3

CGGGCCCATCGTCGCATGCTGATCGCGATTACTAGCATTCCGACTTCATGCAGGCGAGTT-
GCAGCCTGCAATCCGAACTGAGACCGGCTTTTCTAGGATTGGCTCCACCTCGCGGCTTCGTTCCCGTTGTACCGGCCATTGTAGTAC
GTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTGCTTAGAGTGC
CCAGCTTGACCTGCTGGCAACTAAGCATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACA
ACCATGCACCACCTGTCTCCTCTGTCCCGAAGGAAAGCCATATCTCTACAGCGGTCAGAGGGATGTCAAGACCTGGTAAGGTTCTT
CGCGTTGCTTCGAATTAAACCACATACTCCACTGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCC
CCAGGCGGAATGCTTAATGTGTTAACTTCGGCACCAAGGGTATCGAAACCCCTAACACCTAGCATTCATCGTTTACGGCGTGGACTA
CCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGCCCAGAGAGTCGCCTTCGCCACTGGTGTTC
CTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGCTCCCCAGTTTCCAGTGCGACCCG
AAGTTGAGCCTCGGGATTAAACACCAGACTTAAAGAGCCGCCTGCGCGCGCTTTACGCCCAATAATTCCGGACAACGCTTGCCCCC
TACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGGGCTTTCTTCTCAGGTACCGTCACTCTTATAGCAGTTACTCTACAAGACG
TTCTTCCCTGGCAACAGAGCTTTACGATCCGAAACCTTTCATCACTCACGCGGCGTTGCTCCGTCAGGCTTTCGCCATTGGCGGAG
ATTCCCTACTGCTGCCTTCCGGTAGAGTCTGGGCCGGTGTCCTCAGTCCCAAGTGTGGCCGATCACCCTCTTCAGGGTCGGCTACG
CATCGGTCGCCTTGCTAGGCCATTAACCCCACCAACTAGCTATGGCCCGCAGCCAATCCCACAAGGTGACAGATGCTCGGCCTTCC
TCCTTTCCCATGCAGGAAGATGTATCGGGGTATAACCTACCGATTCGGGGTAAGAATACTCCGGTGTCAG

A. Sequence of 16S rDNA amplicon



B. Blastn ouput

NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e value
Accession no.	Name				
AM410706.2	*Acinetobacter grimontii*	2259	98	99	0.0
KF663060.1	*Acinetobacter* sp	2255	98	98	0.0
KF798784.1	Uncultured bacterium clone	2255	98	98	0.0
AB690765.1	Uncultured gamma proteobacterium	2255	98	98	0.0
AB860303.1	*Acinetobacter junii* gene	2255	98	98	0.0

C. Sequence showing homology

Plate 23: Sequence analysis of isolate HPLPSB-3 (*Acinetobacter grimontii*)

Table 30. Cultural and morphological characters of selected actinomycete

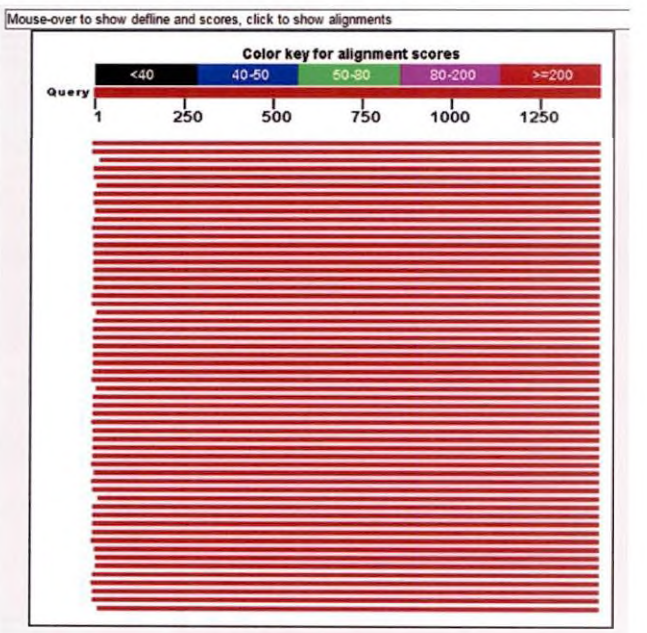
Isolates	Aerial mass color	Substrate mycelium	Melanoid pigment	Gram reaction	Spore chain morphology
HVZACT-2	Light pink	Off white	Absent	+ve	Straight chain

Table 31. 16S rDNA sequence analysis of selected actinomycete

Isolates	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	E value
	Accession no.	Name				
HVZACT-2	HQ680451.1	*Streptomyces termitum*	2259	97	99	0.0
	NR041112.1	*Streptomyces termitum*	2259	97	99	0.0
	EU521702.1	*Streptomyces termitum*	2242	97	99	0.0
	KF48683.1	*Streptomyces viridobrunneus*	2220	97	98	0.0
	FJ006885.1	*Streptomyces* sp.	2220	97	98	0.0

GCTGGTCCCACCTTCGACAGCTCCCTCCCACAAGGGGTTGGGCCACCGGCTTCGGGTGTTACCGACTTTCGTGACGTGACGGGCGG
TGTGTACAAGGCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCAACTCCGACTTCATGGGGTCGAGTTGCAGA
CCCCAATCCGAACTGAGACCGGCTTTTTGAGATTCGCTCCGCCTCACGGCATCGCAGCTCTTTGTACCGGCCATTGTAGCACGTGTG
CAGCCCAAGACATAAGGGGCATGATGACTTGACGTCGTCCCCACCTTCCTCCGAGTTGACCCCGGCGGTCTCCTGTGAGTCCCCATC
ACCCCGAAGGGCATGCTGGCAACACAGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGA
CAGCCATGCACCACCTGTATACCGACCACAAGGGGGCACCCATCTCTGGATGTTTCCGGTATATGTCAAGCCTTGGTAAGGTTCTTCG
CGTTGCGTCGAATTAAGCCACATGCTCCGCTGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCA
GGCGGGGAACTTAATGCGTTTAGCTGCGGCACCGACGACGTGTAATGTCGCCAACACCTAGTTTCCCAAACGTATACGGAGTGGACT
ACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTCGCTCCTCAGCGTCAGTAATGGCCCAGAGATCCGCCTTCGCCACCGGTGTTCC
TCCTGATATCTGCGCATTTCACCGCTACACCAGGAATTCCGATCTCCCCTACCACACTCTAGCCTGCCCGTATCGGATGCAGACCCGG
GGTTAAGCCCCGGGCTTTCACACCCGACGTGACAAGCCGCCTACGAGCTCTTTACGCCCAATAATTCCGGACAACGCTTGCGCCCTA
CGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGCGCTTCTTCTGCAGGTACCGTCACTTGCGCTTCTTCCCTGCTGAAAGAGGTTT
ACAACCCGAAGGCCGTCATCCCTCACGCGGCGTCGCTGCATCAGGCTTTCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTA
GGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGGTCGCCCTCTCAGGCCGGCTACCCGTCGTCGCCTTGGTGGGCCGTTACCCCA
CCAACAAGCTGATAGGCCGCGGGCTCATCCTTCACCGCCGGAGCTTTCCACCCACCCAGATGCGGAGGCGGGTCGTATCCGGTATTA
GACCCCGTTTCCAGGGCTTGTCCCAGAGTGAAGGGCAGATTGCCCACGTGTTACTCACCCGTTCGCCACTAATCCACCCCGAAGGG
CTTCATCGTTCGAC

A. Sequence of 16S rDNA amplicon



B. Blastn output

Isolates	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	E value
	Accession no.	Name				
	HQ680451.1	*Streptomyces termitum*	2259	97	99	0.0
	NR041112.1	*Streptomyces termitum*	2259	97	99	0.0
	EU521702.1	*Streptomyces termitum*	2242	97	99	0.0
HVZACT-1	KF48683.1	*Streptomyces viridobrunneus*	2220	97	98	0.0
	FJ006885.1	*Streptomyces* sp.	2220	97	98	0.0

C. Sequence showing homology

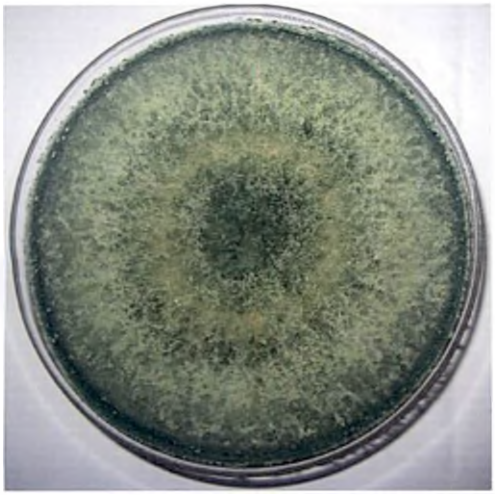
Plate 24: Sequence analysis of isolate HVZACT-2

4.7.3. Identification of selected fungal isolates

The cultural and morphological characters of the two selected fungal isolates were studied in detail on PDA medium. Based on the cultural characters, the isolate FPRF-3 was identified as *Trichoderma* sp. The results obtained are presented in Table 32. Isolates were further identified by National Centre for Fungal Taxonomy (NCFT), New Delhi. NCFT identification was confirmed the isolate FPRF3 as *Trichoderma viride* and HPLF-5 as *Gongoronella butleri* (Plate 25).

Table 32. Identification of selected fungal isolates

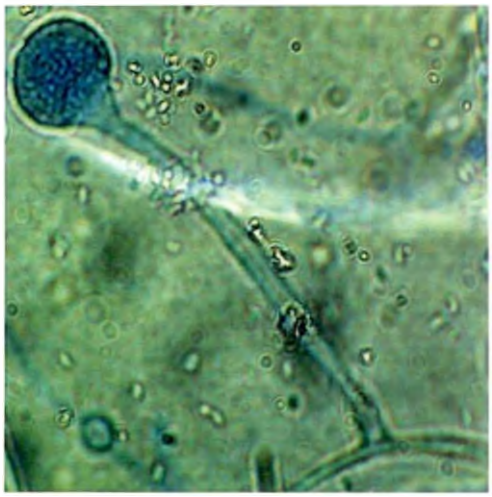
Isolates	**Cultural characters**	**Morphological characters**	**NCFT identification**
FPRF-3	Fast growing and sporulating, mycelium color change from whitish green to dark green and green coloured spores	Branched septate mycelium, green coloured conidia.	*Trichoderma viride*
HPLF-5	Slow growing white turf, dome shaped growth.	Branched mycelium, sporangia globose, sporangiospores oval to flattened on one side	*Gongoronella butleri*



FPRF-3 (*Trichoderma viride*)

Colonies on PDA

Microscopic view
(1000X magnification)



HPLF-5 (*Gongronella butleri*) on PDA

Microscopic view
(1000X magnification)

Plate 25. Colony morphology and microscopic view of PGPM (Fungus)

Discussion

5. DISCUSSION

Soil microorganisms, such as bacteria, fungi and actinomycetes play a central role in maintaining soil fertility and promoting plant health. They are very important as almost every chemical transformation taking place in soil involves active contributions from soil microorganisms. They play an active role in soil fertility as a result of their involvement in the recycling of nutrients like carbon and nitrogen, which are required for plant growth. Soil microorganisms are responsible for the decomposition of the organic matter entering the soil (eg. plant litter) and in the recycling of nutrients in soil. Certain soil microorganisms such as nitrogen fixers and phosphate solubilizers can increase the amount of nutrients present in the soil. Some species of soil microorganisms are used in the microbiological synthesis of antibiotics, vitamins, enzymes and other proteins, aminoacids and gibberellins. Soil microbial populations differ sharply in their optimum pH, temperature, osmotic pressure and source of organic and inorganic matter.

Soil microorganisms probably represent the world's greatest reservoir of biological diversity (Torsvik *et al.,* 1990). The relationship between microbial diversity and function in soil is largely unknown, but biodiversity has been assumed to influence ecosystem stability, productivity and resilience towards stress and disturbance. In some situations, the soil and related plant type may also determine the soil microbial diversity. Root exudates will select for certain populations of microorganisms. Plant roots cause chemical and physical changes to the soil they inhabit and these changes will affect the microbial diversity. In general, soil microbial diversity is related to the complexity of the microbial interactions in soil, including interactions between microorganisms and soil and also microorganisms and plants. The extent of the diversity of microorganisms in soil is seen to be critical to the maintenance of soil health and quality, as a wide range of microorganisms is involved in important soil functions.

Plant rhizosphere is known to be preferred ecological niche for various types of soil microorganisms due to rich nutrient availability. Rhizosphere supports large and active microbial population capable of exerting beneficial, neutral and detrimental effects on the plants (Atlas and Bartha, 1998). The plant root-soil interface is a dynamic region in which numerous biogeochemical processes take place driven by the physical activity, chemicals released by the plant root and mediated by soil microorganisms. The cocktail of chemicals released is influenced by plant species, edaphic and climatic conditions which together shape the microbial community within the rhizosphere. Changes to the physical, chemical and biological properties of rhizosphere soil have significant influence on the subsequent growth and health of plants. Many plants exhibit genetic resistance or tolerance to rhizosphere microorganisms; the variety of plant will determine the community makeup of the microorganisms in the rhizosphere. The age and health of the plants play a role in the microbial community dynamics of the rhizosphere. Plants can also compete with rhizosphere microorganisms for resources like water and nutrient (Sylvia, 2005). In general, plant bacterial interactions in the rhizosphere are the determinants of plant health and soil fertility.

Microorganisms that exert beneficial effects on the growth of the host plant via direct or indirect mechanisms are termed as plant growth promoting microorganisms (PGPM). Correspondingly, the bacteria that colonize rhizosphere and promote plant growth are termed as PGPM. PGPM includes beneficial groups such as growth promoters, antagonists that colonizes the roots of plant and promote growth by several mechanisms. In the last few years, the number of PGPM that have been identified has greatly increased, mainly because the role of the rhizosphere as an ecosystem has gained importance in the functioning of the biosphere. Beneficial plant-microbe interactions in the rhizosphere can influence plant vigor and soil fertility. These beneficial effects of PGPM have direct or indirect effect on the growth and yield of plants. Direct promotion of growth by PGPM including production of

metabolites that enhances plant growth such as auxins, cytokinins, gibberellins and through solubilization of phosphate minerals (El-Hadad *et al.*, 2010). Indirect growth promotion occurs via the removal of pathogens by the production of secondary metabolites such as hydrogen cyanide and siderophores.

Black pepper (*Piper nigrum* L), a highly valued spice crop, is a perennial vine which originated in the Western Ghats of India. It is one of the oldest spices known to mankind. The production of black pepper is limited by many diseases. Foot rot and yellowing are the most important diseases affecting black pepper production in Wayanad district. Foot rot is caused by the soil-borne fungal pathogen *Phytophthora capsici*. Since the pathogen is soil borne, native antagonistic microorganisms play a major role in keeping the population of pathogen at low levels. Yellowing is a debilitating disease of black pepper. Foliar yellowing, defoliation and die back are the aerial symptoms of this disease. The exact cause of yellowing is unknown. It is considered as a complex disease caused by infestation of plant parasitic nematodes, soil borne pathogens and nutrient imbalance. However, some plants in the diseased garden affected by either foot rot or yellowing, remain healthy. This may be due to the inherent genetic ability of the plant or by the effect of rhizosphere microflora on their growth. Increased photosynthetic rates in healthy plants may result in increased root exudation, which may in turn, contribute to the proliferation of microorganisms in the rhizosphere of such plants.

To assess the diversity of beneficial microorganisms with respect to plant growth promotion and disease management, it is necessary to compare microbial communities inhabiting the rhizosphere of both healthy and diseased plants. Beneficial microorganisms present in the rhizosphere of healthy plants could be exploited for biological control of plant diseases. No systematic studies have been undertaken on the diversity of rhizosphere microflora with respect to foot rot or yellowing diseases in black pepper. Hence, the present study was taken up to assess the functional diversity of beneficial microflora from the rhizosphere of black pepper

in Wayanad district, with respect to their plant growth promoting and antagonistic activities.

In this study, the population of different groups of microorganisms such as bacteria, fungi and actinomycetes including beneficial microbes like nitrogen fixers, phosphate solubilizers, *Bacillus* sp. and fluorescent pseudomonads was enumerated on 16 different media. Different media were used because a single medium may not be optimum for all microorganisms present in the soil. The purpose of using different media was to obtain maximum number of isolates from the soil. Oligotrophic media are those which contain low concentration of nutrients and in the present study media containing 50 per cent concentration of the original media were used. Twelve soil samples consisting of four each from healthy, foot rot affected and yellowing affected gardens in Wayanad district. Results revealed that the highest population of bacteria ($16.6x10^6$ cfu g^{-1}) was recorded in rhizosphere soils of healthy plants of Perikallur (HPR). Rhizosphere soil from healthy garden of Pulpulli (HPL) recorded highest population of fungi ($6.0x10^4$ cfu g^{-1}), phosphate solubilizers ($9.3x10^5$ cfu g^{-1}) and fluorescent pseudomonads ($2.5x10^3$ cfu g^{-1}). This may be due to the rich source of mineral nutrients in the healthy rhizosphere, which directly influence the microbial diversity and harbour high population of beneficial groups than the disease affected soils. Specific root exudates secreted by the plant species may also select specific beneficial groups. However, in the present study, higher population of actinomycetes and nitrogen fixers was observed in the rhizosphere soils of yellowing affected garden followed by healthy gardens.

Goudar and Kulkarni (2000) reported a higher population of bacteria and actinomycetes from the rhizosphere of healthy pigeon pea than the wilt affected ones. They proposed that this could be due to the fact that the diseased roots supported a larger population of *Fusarium* which might have competitively suppressed the bacteria and actinomycetes. This is similar to the findings of Pandey and Upadhyay (2000), who observed that rhizosphere of diseased and healthy plants harbor several

types of fungi and bacteria, which differ in their behavior. This is also in conformity with the study of Sharma *et al.* (2010), who reported higher population of beneficial microflora in rhizosphere and rhizoplane of healthy mulberry gardens compared to diseased gardens. This indicated that the population of beneficial microflora was not affected in healthy gardens, as the pathogens causing soil borne diseases in mulberry were not encountered. However, some earlier reports indicated that the size of culturable bacterial populations, especially fluorescent pseudomonads, was larger in the rhizosphere of wheat infected with *Guammanomyces graminis* var. *tritici* (Sarniguet *et al.,* 1992). In a similar study by Gardener and Weller (2001) observed higher population of fluorescent pseudomonads in the rhizosphere of wheat infected with *G. graminis* var. *tritici* (Take-all pathogen) when the plants were grown in growth chambers. This was further confirmed by generating fluorescence-tagged amplified rDNA restriction analysis (FT-ARDRA). They also reported that population of *Chryseobacterium* and *Flavobacterium* was also more in infected plants.

Plant growth promoting microorganisms (PGPM) are a heterogeneous that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and/ indirectly. PGPM have been reported to directly enhance plant growth by a variety of mechanisms: fixation of atmospheric nitrogen, solublization of minerals such as phosphorus, production of siderophores, and synthesis of plant growth hormones ie. indole-3- acetic acid (IAA), gibberellic acid, cytokinins, and ethylene. Indirect mechanisms involves the biological control of plant pathogens and deleterious microbes, through the production of antibiotics, lytic enzymes, hydrogen cyanide, catalase and siderophore or through competition for nutrients and space can improve significantly plant health and promote growth, as evidenced by increases in seedling emergence, vigor, and yield. Production of indole-3-ethanol or indole-3-acetic acid (IAA), the compounds belonging to auxins, have been reported for several bacterial genera. PGPM also help

in solubilization of mineral phosphates and other nutrients, enhance resistance to stress, stabilize soil aggregates, and improve soil structure and organic matter content. PGPM retain more soil organic N, and other nutrients in the plant-soil system, thus reducing the need for fertilizer N and P and enhancing release of the nutrients.

IAA is one of the most important phytohormones and functions as an important signaling molecule in the regulation of plant development. It is one of the most physiologically active auxins plays a key role in the regulation of plant growth and development. Many bacteria isolated from the rhizosphere of different crops have a greater potential to synthesis IAA *in vitro* in the presence and absence of physiological precursors, mainly tryptophan. Microbial isolates from the rhizosphere of different crops have the capacity to synthesize and release IAA as secondary metabolites because of the relatively rich supply of substrates.

In the present study, 207 isolates including bacteria, fungi and actinomycetes were screened for IAA production on the basis of development of pink colour in presence of tryptophan. Ten isolates which were positive in the preliminary screening for IAA production were used for quantitative estimation under *in vitro*. Quantity of IAA production varied with isolates and the isolate HPLBC-6 (*Providencia* sp.) recorded highest IAA production of 292.50 $\mu g\ ml^{-1}$ in the presence of L-tryptophan. Previous studies on biological nitrogen fixation by Chaiharn *et al.* (2008) revealed the role of IAA produced by rhizobacteria in increasing the absorption of nutrients by increasing the production of root hairs by the plant. Another report by Patten and Glick (1996) revealed that 80% of bacteria isolated from the rhizosphere could produce IAA. Similarly, higher level of IAA production by *Pseudomonas* and *Klebsiella* was reported by other research workers (Xie *et al.*, 1996; Chaiharn and Lumyong, 2011). It has been reported that IAA production by PGPR can vary among different species, strains, culture conditions, growth stage and substrate availability (Mirza *et al.*, 2001). The production of IAA was found dependent upon bacterial isolates and concentration of tryptophan. Such findings may have direct practical

application, although intrinsic ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant.

Phosphorus (P) is one of the major essential macronutrients for plant growth and development. Phosphorus exists in two forms in soil, as organic and inorganic phosphates. To convert insoluble phosphates (both organic and inorganic) compounds in a form accessible to the plant is an important trait for a PGPM in increasing plant yield. The existence of soil microbes capable of transforming soil phosphorus and fixing nitrogen from the atmosphere to available forms has been recorded by many investigators. It has been reported that high proportion of phosphate solubilizing microorganisms are concentrated in the rhizosphere of plants.

Preliminary screening for phosphate solubilization efficiency of the isolates was carried out by the formation of clear zone on agar media and solubilization efficiency was calculated by the semiquantative method (Nguyen et al., 1992). The diameter of solubilized clear zone ranged from 0.9mm (YPTPSB-1) to 5.8 mm (HPLF-5). In a similar study using rhizospheric soils of French bean, Kumar *et al.* (2012) observed that *diameter* of clear zone ranged from 4 to 20 mm, for different isolates. In the present study, solubilization efficiency ranged from 102.7 per cent (HPLF-1) to a maximum of 216.7 per cent (HPLPSB-3), later identified as *Acinetobacter grimonti*. Quantitative estimation of solubilized P by the phospho-molybdic blue colour method revealed that HPLPSB-3 was the most efficient one, with 162.7 $\mu g\ ml^{-1}$, in a period of two weeks. This was followed by HPLF-5 (*Gongoronella butleri*) which solubilized about 161.3 $\mu g\ ml^{-1}$ of phosphorus. Several workers have previously quantified phosphate solubilization of microorganisms by the same method and a few of them reported *Acinetobacter* sp. as efficient solubilizers. Kang *et al.* (2009) demonstrated the *in vitro* phosphate solubilization potential of *Acinetobacter calcoaceticus* SE370 over a period of 6 days on agar media. An increase in clear zone with time suggested an increase in acid secretion and thus, conversion of insoluble phosphate to soluble form. Ogut et al. (2010)

reported that out of a total of 21 PSM isolated from the rhizosphere soil of wheat and maize, *Acinetobacter* strains were the most effective solubilizers of tricalcium phosphate and the mean P dissolved in liquid cultures of *Acinetobacter* strains in a 5-day incubation ranged from 167 to 888 µg/ml P. Similarly, Chaiharn and Lumyong (2011) screened 216 rhizobacterial strains for their ability to solubilize tricalcium phosphate in PVK broth. The results indicated that the P solubilizing ability of test isolates varied from 0.50 to 334.4 µg/ml. Among these isolates *Acinetobacter* strain CR 1.8 had the highest P solubilization of 334 ± 0.4 µg/ml.

Reduction of pH of the media indicates that the production of acids was most likely to be responsible for phosphate solubilization. The amount of P solubilized may be highly dependent on the source of the P and to a large extent by the culture conditions. Similar studies reviewed by Kucey *et al.* (1989) pointed that microbial solubilization of soil phosphate in liquid medium studies has often been due to the excretion of organic acids as a result of which a decrease in pH was effected. Current results confirmed the previous report of (Kpomblekou and Tabatabai, 1994) that the microorganisms which decrease the medium pH during the growth are efficient P solubilizers. Ogut *et al.* (2010) also reported that the pH dropped to below 4.7 from 7.8 in six strains of *Acinetobacter*.

The recognition in mid-70s that biological nitrogen fixation offers the most promising supplement to chemical nitrogenous fertilizers led to a wide array of studies on associative, free living and symbiotic nitrogen fixation by rhizosphere microflora. These microbes are commonly found in association with the roots of diverse plants. In the present study, 38 nitrogen fixers were screened for their nitrogen fixation on N-free medium and five were further selected based on their growth. Among the five selected isolates, YPTN-3 (*Paenibacillus* sp.) which recorded excellent growth on nitrogen free agar medium fixed maximum amount of nitrogen (46.92 mg) per gram of sucrose utilized. More than 16 species of bacteria have been reported to possess nitrogen fixing ability. The genus *Paenibacillus* was

earlier described by Ash *et al.* (1993) on the basis of analysis of the 16S rRNA gene sequences of group 3 *Bacilli*. Members of the genus are widely distributed in nature and have diverse physiological characteristics. Nitrogen fixation has been described in some species of *Paenibacillus*, such as *P. polymyxa, P. macerans, P. azotofixans, P. peoriae, P. graminis, P. odorifer* and *P. brasiliensis* (Wied *et al.*, 2002). Some species consistently showed a great capacity to fix atmospheric nitrogen *in vitro* (Berge *et al.*, 2002). It has been reported that *P. azotofixans* has the highest nitrogenase activity among these nitrogen fixers. Similarly, Ding *et al.* (2005) first demonstrated the ability of nitrogen fixation by *B. marisflavi* and *P. massiliensis* . They were also detected *nifH* gene fragement in both of these genera by designing degenerate primers. Ma *et al.* (2007) reported nitrogen fixation by a novel species of *Paenibacillus* viz. *P. zanthoxyli*, which was identified by 16S rRNA gene sequencing. They also detected the nitrogen fixation by acetylene reduction and from the presence of the *nifH* gene in *Paenibacillus sabinas* isolated from the rhizosphere soil of a shrub (*Sabina squamata*).

Earlier reports have indicated that PGPM enhance seed emergence, growth, crop yield, and contribute to the protection of plants against certain pathogens and pests. In the present study, six selected PGPM were evaluated for growth promotion in black pepper seedlings. The results of *in planta* experiment indicated that PGPM enhanced sprouting of black pepper cuttings. Observations recorded after one month of planting revealed that T_1 (YPTN-3, *Paenibacillus* sp.) produced the maximum sprouting (33.3 per cent). This isolate was found to efficiently fix atmospheric nitrogen (46.92 mg N/ g sucrose utilized) and also produce IAA (19.88μg ml^{-1}). However, after three months of planting T3 (*Acinetobacter grimontii*) recorded maximum sprouting of 83.33 %. The intake of minerals and production of plant hormones could also help in the early establishment of pepper cuttings. Earlier studies revealed that the strains of *Pseudomonas* could solubilize complex forms of P in soil thus making it available to the plant. According to Diby *et al.* (2005), the

intake of minerals such as N and P was found to be more with *P. fluorescens* treated black pepper plants. Kumar *et al.* (2012) found that five bacterial isolates *Acinetobacter* sp., *Pseudomonas* sp., *Enterobacter* sp., *Micrococcus* sp., and *Bacillus* sp. positively affected the germination of *Pisum sativum* and *Zea mays*.

Other biometric characters were also recorded in the *in planta* experiment. The treatment, T3 (HPLPSB-3, *Acinetobacter grimontii*) recorded maximum shoot length, number of leaves at four and six months after planting. This isolate was found to be the most efficient among all phosphate solubilizers, in the *in vitro* screening and quantification of phosphate solubilization. The same isolate was also found to produce IAA to the extent of 24.02µg ml^{-1}. It is possible that the phosphate solubilization and auxin production might have improved plant growth both by hormonal stimulation and by supplying phosphate. These results are in agreement with the report of Anith *et al.* (2002) that the treatment of the cuttings with *Pseudomonas fluorescens* PN-026 resulted in increased shoot growth compared to the uninoculated control. Similar studies conducted by Ramachandran *et al.* (2003) indicated that the PSB strain (PB-21) improved the shoot and root growth of the black pepper cuttings efficiently by solubilization and release of P from insoluble rock phosphate. There are also reports where soil inoculation with P solubilizing bacteria significantly increased N and P uptake in black pepper and cucumber plants (Han *et al.*, 2006). Similarly, Henri *et al.* (2008) reported that the plant inoculation with P solubilizing *Pseudomonas fluorescens* enhanced the growth and yield of maize. Thus inoculation with phosphate solubilizers made more soluble phosphates available to the growing plants. This may be the reason for improved growth and yield of the maize plants. In an another study, Leaungvutiviroj (2010) reported the application of biofertilizer consortium containing nitrogen fixer (*Azotobacter tropicalis*), phosphate solubilizing bacteria (*Burkholderia unamae*), potassium solubilizer (*Bacillus subtilis*) and an auxin producer (KJB9/2 strain) enhanced the growth and yield of corn and vegetables.

In the present investigation, maximum number of roots per seedling (11.00) was observed in T_3 (HVKNF-6, *Paenibacillus* sp.). Maximum root length (19.13cm) was observed in T_5 (HPLBC-6, *Providencia* sp.) whereas, maximum fresh root weight was in T_3 (HPLPSB-3, *Acinetobacter grimontii*). The intake of minerals, nutrients and production of growth factors by the bacterial isolates might be the reason for increased root number, length and weight. These factors not only stimulated roots for higher absorbtion of minerals but also results in better root biomass. There is evidence that the growth hormones produced by the bacteria can in some instances increase growth rates and improve yields of the host plants. Most root promoting bacteria synthesis IAA and it has been clearly demonstrated that they stimulated the formation of lateral and adventitious roots (Barbieri and Galli, 1993). The other evidences include that the *P. putida* GR 12-2 cells that produced wild type levels of IAA stimulated the formation of many short adventitious roots on mung bean cuttings and in IAA over producing mutant stimulated the formation of even more adventitious roots than the wild type strain (Mayak *et al.,* 1997). The present findings are in line with Ramachandran *et al.* (2003) who observed maximum production of finer roots in black pepper treated with microbial inoculants than the uninoculated control.

Pepper leaves showing typical symptoms of foot rot were collected from the pepper gardens of College of Horticulture, Vellanikkara. Pathogen was isolated from the collected specimen by culturing it on carrot agar (CA) medium. Streptomycin was provided in the CA medium to avoid bacterial contamination which also initially inhibited the mycelial growth of the pathogen. This may be because the pathogen *Phytophthora capsici* is highly susceptible to streptomycin. Streptomycin is a member of aminoglycosides which is one of the major classes of antimicrobial agents. Similarly, Cohen and Perl (1973) also found the mycelial inhibition of *Phytophthora infestans* in wheat seed medium containing 10 µg/ml of streptomycin.

They also reported that streptomycin has directly inhibited the zoospores release of the pathogen.

Foot rot pathogen *P. capsici* was identified by cultural characters and pathogenicity proved by proving Koch postulates, using detached, healthy pepper leaves.

The antagonistic activity of biocontrol microorganisms is often demonstrated by the inhibition of mycelial growth or a reduction in symptoms of infected plants. In this study, a total of 112 bacterial isolates were screened for their antagonistic activity against *P. capsici*. Among these, HPLPSB-6 (*Paenibacillus polymyxa*) and HABB-1 recorded per cent inhibition of 69.27 and 65.00 respectively and scored the first rank with respect to per cent inhibition. Several authors have also reported the results of dual culture assay that agree with the present findings. Jubina and Girija (1998) used several bacterial antagonists against *P. capsici* in black pepper after initially screening the isolates by dual culture. Their studies on already established nursery plants of black pepper revealed that one isolate of Bacillus (B13) showing poor inhibition of the fungal pathogen in the dual culture exhibited the highest disease suppression in the *in vivo* biological control assay. This is corroborated by the study of Rubio *et al.* (2000) in which *P. fluorescens* inhibited *Phytophthora infestans* to an extent of 74%. They also reported a clear zone of inhibition in the dual plate is suggestive of production of antagonistic metabolites by *P. fluorescens*. Anith *et al.* (2002) screened 64 bacterial isolates against *P. capsici* and isolates PN032 and *Pseudomonas* sp. showed high inhibition of fungal growth in dual culture studies. It has been further tested in shoot assay and *Pseudomonas* sp. showed maximum lesion suppression, but PN-032 which represented high inhibition in dual culture and poor inhibition in shoot assay. Thus, the ability of the antagonists to suppress the lesion in shoot assay could be correlated with their biological control potential. Narasimhan and Sivakumar (2012) reported that the *Bacillus* sp. from the chilli rhizosphere showed broad spectrum antagonism against *Alternaria* sp. (55%), *Colletotrichum*

gloeosporioides (57%), *Phytophthora capsici* (62%), *Rhizoctonia solani* (42%), *Fusarium solani* (42%), *Fusarium oxysporum* (40%) and *Verticillium* sp. (36%).

In the present study, out of 32 actinomycetes screened for their antagonism against *P. capsici* by dual culture method *in vitro*, seven isolates inhibited mycelial growth of *P.capsici* in the range of 62.50% to 66.66% and scored the first rank. Among these isolate HVZACT-1 (*Streptomyces termitum*) gave maximum inhibition of 66.66%. Mycelial inhibition of *P. capsici* may be due to the production of antifungal compounds by the antagonist.

The present study, is in agreement with (Lim *et al.*, 2007) who demonstrated that the *S. padanus* strain TH-04 produced valinomycin, possessing antifungal activity against *P. capsici*. Similarly, Xiong *et al.* (2012) found that *Streptomyces padanus* strain JAU4234 possess antimicrobial activity against various Gram-positive and Gram-negative bacteria and fungi. They were also found that the strain produces an antifungal antibiotic fungichormin and an antitumor antibiotic like actinomycin X_2. Nguyen *et al.* (2012) reported that *Streptomyces griseus* H7602 from the rhizosphere soil recorded an inhibiton of 53.33% on *P. capsici* causing root rot of pepper (*Capsicum annuum*) and produced active substance as well as several lytic enzymes including chitinase and β-1,3-glucanase *in vitro*. This is in line with the study of Chi *et al.*(2013) who reported the *in vitro* inhibition of mycelial growth and zoosporangium formation of *P. capsici* causing *Phytophthora* blight on pepper plants (*Capsicum annuum*) by *S. padanus*.

In the present investigation, sixty three fungal isolates were also evaluated for their *in vitro* antagonistic activity against *P. capsici* by dual culture method. These isolates exhibited inhibition in the range of 9.59% to 100%. The isolate FPRF-3 *Trichoderma viride*, obtained from foot rot affected garden recorded maximum mycelial inhibition of 100%. Maximum efficiency of the fungal antagonists may be

due to the fastest mycelial growth of the antagonists over the pathogen or due to the production of antibiotics or other lethal compounds.

Trichoderma spp. have already been reported to be antagonistic to several soil borne fungal pathogens, through various mechanisms including mycoparasitism, production of secondary metabolites and lytic enzymes. Weindling (1932) reported the mycoparasitism of *Rhizoctonia solani* causing damping off disease by the hyphae of *Trichoderma*. He also described production of an antibiotic by the antagonist which was toxic to both *R. solani* and *Sclerotinia americana* and named it as gliotoxin. Rajan *et al.* (2002) isolated a number of *Trichoderma* isolates and were screened both *in vitro* and *in vivo*. Isolates *T. virens*-12 and *T. harzianum*-26 were found to be more effective in controlling foot rot disease. Shashidhara *et al.* (2008) reported that *Trichoderma* sp. inhibited 58.41 % mycelial growth of *P. capsici* isolated from black pepper, in dual culture test.. Similarly, Kavitha and Nelson (2013) reported antagonism of *Trichoderma viride* and *Trichoderma koningii* isolated from the rhizosphere soil of sunflower. In dual culture method, *Trichoderma viride* recorded maximum growth inhibition of 72.20% against *Fusarium oxysporum* whereas, *Trichoderma koningii* effectively inhibited *Pythium debarianum* (57.42%).

Understanding the mechanisms of antagonism is critical to the eventual improvement and effective use of biocontrol agents. The widely recognized mechanisms of biocontrol mediated by PGPR includes competition for an ecological niche, production of inhibitory allelochemicals and induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens.

In the present investigation, mechanism behind antagonistic activity such as HCN, ammonia and siderophore production was assessed for selected antagonistic bacteria, actinomycetes and fungi obtained from Wayanad. All the selected bacterial isolates were found to produce HCN, whereas none of the fungal antagonists were found to be positive. Among the bacterial antagonists, HPLPSB-6 (*Paenibacillus*

polymyxa), HABB-1 and HABB-1 were found to be good HCN producers. Out of seventeen actinomycetes selected, eleven were confirmed to be HCN producers. Among the actinomycetes selected, antagonist HVZACT-1 (*Streptomyces termitum*) was found to be a moderate HCN producer. This isolate was found to inhibit the growth of *P. capsici*, most efficiently among all actinomycetes tested under *in vitro* conditions and hence the mechanism of antagonism could be attributed to HCN production. Earlier reports by Voisard *et al.* (1989) indicated that production of HCN by *Pseudomonas fluorescens* resulted in suppression of soil borne pathogens. Similarly, Flaishman *et al.* (1996) reported that overproduction of HCN may control many fungal diseases in wheat seedlings. Paul *et al.* (2005) studied the antagonistic action of 12 fluorescent pseudomonads against *P. capsici.* These 12 isolates were found to supress the pathogen through different modes namely, production of volatile and non volatile inhibitory metabolites including HCN and siderophore mediated mechanism. Kumar *et al.* (2008) also reported *in vitro* antagonism by HCN producing PGPR against sclerotia germination of *M. phaseolina.* Ryall *et al.* (2009) also reported production of secondary metabolite HCN by an entomopathogenic bacterium *Pseudomonas entomophila* and it was the main implication for biocontrol properties and pathogenecity exerted by other bacteria. Vanitha and Ramjegathesh (2014) screened the *P. fluorescens* strains against *Macrophomina phaseolina* (Tassi) Goid, the causal organism of coleus root rot. The results revealed that strain Pf1 recorded maximum inhibition of mycelial growth. They also found out the mechanisms behind the antagonism were HCN, siderophore and antibiotic production.

Selected antagonists were tested for ammonia production which is another attribute in the mechanism of antagonism. Among the seven bacterial antagonists tested for ammonia production, HPLPSB-6 (later identified as *Paenibacillus polymyxa*) and HABBC-2 obtained from healthy gardens were found to be good ammonia producers. Among the selected actinomycetes, five isolates (HVZACT-1

[*Streptomyces termitum*], HPLACT-4, FPTACT-1, FPTACT-2 and FPRACT-1) were found to be good ammonia producers. All the four fungal antagonists tested were found to be weak producers of ammonia. Production of ammonia has been reported to be a mechanism of antagonism by several workers. Martin (1982) reported that the accumulation of ammonia in soil may increase in pH creating alkaline condition of soil at pH 9-9.5. This condition suppresses the growth of certain fungi and nitrobacteria due to its potent inhibition effect. He also reported that this will upset the microbial community and inhibit germination of spores of many fungi. Trivedi *et al.* (2008) isolated *Pseudomonas corrugata*, a soil bacterium originally from a temperate site of Indian Himalayan Region (IHR) and examined for its antagonistic activities against two phytopathogenic fungi, *Alternaria alternata* and *Fusarium oxysporum*. They also observed production of ammonia by the isolate which could be responsible for its antagonistic action.

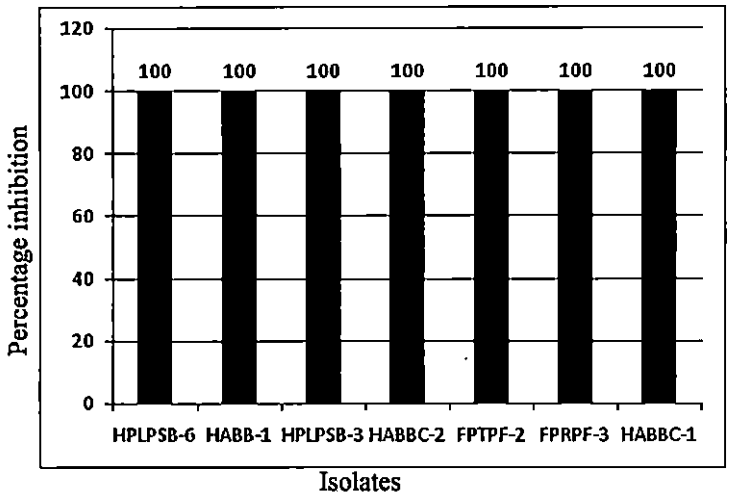
Siderophore production is yet another mechanism behind antagonism. These are low molecular weight, iron chelating compounds secreted by microorganisms such as bacteria, fungi and actinomycetes. They bind to the available form of iron in the rhizosphere, thus making it unavailable to the phytopathogens and protecting the plant health.

In the present study production of siderophore by the selected antagonists were detected by the formation of yellow-orange zone in CAS medium. Among seven bacterial antagonists tested, three were (HPLPSB-6: *Paenibacillus polymyxa*, HPLPSB-3: *Acinetobacter grimontii* and FPTPF-2) positive for siderophore production. Likewise five actinomycetes (HVZACT-1, HPLACT-2, HVZACT-3, HPLACT-4 and FPTACT-1) were also confirmed as siderophore producers. Fungal antagonists HPLF3 and FPRF3 (*Trichoderma viride*) were also positive for siderophore production. Production of siderophore by the antagonists might be also responsible for the inhibition of mycelial growth of *P. capsici*. Hien *et al,* (1992) reported that siderophore production is involved in the antagonistic effect of

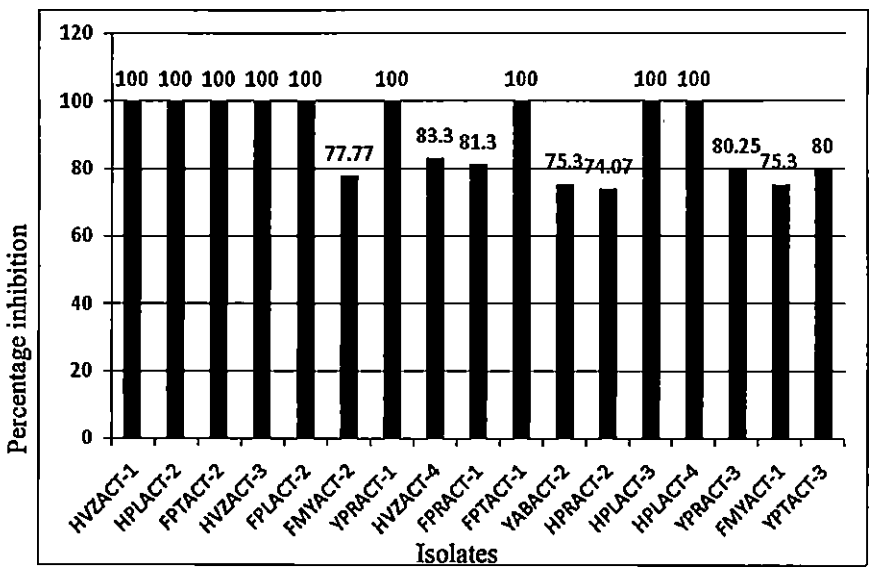
Pseudomonas aeruginosa strain 7NSK2 against *Pythium* causing post emergence damping off. Tian *et al.* (2009) studied the distribution of siderophore producing isolates according to amplified ribosomal DNA restriction analysis (ARDRA) groups and revealed that most of the isolates belonged to the Gram- negative bacteria *Pseudomonas* and *Enterobacter* genera, and Gram-positive bacteria included *Bacillus* and *Rhodococcus* genera.

In the present study, effect of culture filtrate of selected antagonists on radial growth of *P. capsici* was assessed as an indication to the production of non-volatile compounds. Culture filtrate from all the seven bacterial antagonists (HPLPSB-6: *Paenibacillus polymyxa*, HABB-1, HPLPSB-3 and HABBC-2 and FPTPF-2) inhibited the full growth of *P. capsici* with an inhibition of 100 per cent. Among the actinomycetes, ten isolates viz. HVZACT-1 (*Streptomyces termitum*), HPLACT-2, HVZACT-3, HPLACT-3, & HPLACT-4, FPLACT2, FPTACT-1, FPTACT-2 & FMYACT-2 and YPRACT-1 recorded 100 per cent inhibition on the mycelial growth of *P. capsici*. Fungal antagonists FPRF-3 (*Trichoderma viride*) and HPLF-3 recorded 100 per cent inhibition on the mycelial growth of the pathogen. The inhibition of pathogen in the assay might be due to the production of diffusible compounds related to secondary metabolism, hydrolytic enzymes or both of them. Similar results of inhibition of radial growth of *Fusarium graminearum* by non volatile metabolites produced by *Trichoderma hamatum* T612 was reported by Hajieghrari *et al*, (2008). Hasan *et al.* (2009) reported that the production of viridiofungin A in culture filtrate of *T. harzianum* isolate T23 prevented germination of *Phytophthora infestans* sporangia. Similarly, Adebola and Amadi (2010) observed that the culture filtrate of the test fungus *Paecilomyces lilacinus* inhibited the growth of *Phytophthora palmivora*.

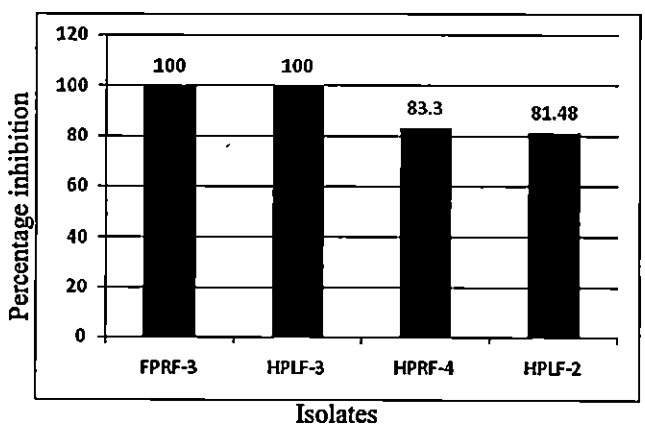
Mycoparasitism is yet another mode of antagonism by the fungal antagonist in controlling the growth of pathogen. The complex process of mycoparasitism consists of several events, including recognition of the host, attack and subsequent penetration



A. Bacteria



B. Actinomycetes



C. Fungi

Fig. 1. Effect of culture filtrate on radial growth of *P. capsici*

and killing. In the present investigation, the selected four fungal antagonists were also observed microscopically for their hyphal interaction from the dual culture plate. All the four fungal antagonists were found to intermingle and coils around the the hyphae of *P. capsici*. Antagonist FPRF-3 (*Trichoderma viride*) and HPRF-4 (which gave 75.17 % and 62.50 % respectively in dual culture were found to coil around and finally lysing the hyphae of *P. capsici* (Plate 14 D). Intermingling and coiling by antagonists along with the production of volatile and non-volatile metabolites might be the major mechanism behind the mycelial inhibition of *P. capsici* by *Trichoderma viride*. In agreement to this result, Pugeg and Ian (2006) reported the mycoparasitic action of *Trichoderma* strains against *Phytophthora cinnamoni*. Several workers reported that during this process *Trichoderma* secretes CWDEs (cell wall degrading enzymes) that hydrolyze the cell wall of the host fungus, subsequently releasing oligomers from the pathogen cell wall (Kubicek *et al.*, 2001; Howell 2003; Woo *et al.*, 2006). Similarly, Zegeye *et al.* (2011) examined the hyphal interactions between *T. viride* and the *P. infestans* and revealed that *T. viride* hyphae coiled around and grew along the hyphae of *P. infestans*.

Three most efficient PGPM selected from *in planta* screening for growth promotion and three most promising antagonists selected based on *in vitro* antagonistic activity were evaluated *in planta*. The selected PGPM included YPTN-3 (*Paenibacillus* sp.), HPLPSB-3 (*Acinetobacter grimonti*) and HPLBC-6 (*Providencia* sp.). HPLPSB-6 (*Peanibacillus polymyxa*), HVZACT-1 (*Streptomyces termitum*) and FPRF-3 (*Trichoderma viride*) were the antagonists for *in planta* evaluation. *Pseudomonas fluorescens* and *Trichoderma viride* commercialized by KAU as biocontrol agents were used as reference cultures.

Incidence and severity of foot rot disease in the experimental plants were recorded for each treatment. Maximum disease incidence was observed in T_9 (control with pathogen alone). Disease incidence was recorded minimum (6.23%) in T_6 [FPRF-3 (*Trichoderma viride*)] and this was followed by T_5 [HVZACT-1

(*Streptomyces termitum*)] with 13.20%. In case of per cent disease severity (PDI) also, least severity (4.00 %) was observed in T_9 [FPRF-3 (*Trichoderma viride*)]. The isolate, FPRF-3 recorded maximum mycelial inhibition of *P. capsici* (100%) in dual culture. This isolate had recorded production of NH_3 and siderophore under *in vitro* experiments. The same isolate had also recorded 100 per cent inhibition of the fungal pathogen when the culture filtrate was incorporated in the medium, indicating the presence of non-volatile metabolites capable of inhibiting the pathogen. Similarly, Sarma *et al.* (1994) reported *Trichoderma* as efficient biological control agent against *P. capsici* induced root rot in black pepper. *T. harzianum* and *T. viride* have been used as biological control agents against diseases of black pepper caused by *P. capsici* both in main field and in nursery (Anandaraj and Sarma, 1995). This is in line with the reports of Rajan *et al.* (2002) who reported the effectiveness of *T. virens*-12 and *T. harzianum*-26 in controlling foot rot disease in black pepper. They also reported that when mixtures of different *Trichoderma* isolates were used, the infection was not reduced. This was attributed to competition among the isolates which might have nullified their individual effects. Several authors (Harman *et al.*, 2004; Woo *et al.*, 2006; Woo and Lorito, 2007) have reported that during interaction of *Trichoderma* with the plant, different classes of metabolites may act as elicitors or resistance inducers. These molecules include proteins with enzymatic activity, avirulence like gene products able to induce defence reactions in plants and low molecular weight degradation compounds from fungal cell wall. These compounds elicited a reaction in plant when appiled to plant. Further these were also stimulated the biocontrol ability of *Trichoderma* by activating the mycoparasitic gene expression cascade. In a study by Zegeye *et al.* (2011), *Trichoderma viride* was found to be more efficient than *P. fluorescens* in controlling *Phytophthora infestans* causing late blight of potato.

Plant growth parameters were also recorded separately for each treatment. It was found that length of the vine was significantly increased at 30 days as well as 60

days after the application of bioinoculants, in plants treated with bacterial isolates HPLBC-6 and HPLPSB-6; actinomycete HVZACT-1 and fungal isolate FPRF-3, when compared to uninoculated control. Reference culture *Trichoderma viride* also recorded significantly. Maximum vine length was recorded by FPRF-3. Number of leaves were found to be maximum in T_6 (FPRF-3, native isolate of *Trichoderma viride*) at 30 and 60 days after bioinoculant application.

Maximum number of roots (7.53) and fresh weight of roots (0.88 g) were obtained in T_6 treated with FPRF-3 (*Trichoderma viride*), whereas maximum root length was observed in T_4 treated with HPLPSB-6 (*Paenibacillus polymyxa*). Production of volatile compounds and non- volatile compounds by the isolate FPRF-3 which was confirmed in *in vitro* assay might have attributed for the vigour of the seedlings. Interaction of the antagonists with the plant roots might have also induced production of some compounds which help in plant growth and development. Similarly, Cutler *et al.* (1986) reported the secondary metabolites produced by the *Trichoderma* sp. acted as plant growth regulators. They also suggested that these secondary metabolites may act as auxin like compounds. Chang *et al.* (1986) and Mukhopadhyay (1988) reported increased growth response of several plants in the presence of biocontrol agents, which may be caused by a direct effect on the plant as a biofertilizer. Singh *et al.* (2008) reported that in addition to red rot control in sugarcane, *T. harzianum* and *T. viride* improved the germination, number of tillers, number of millable canes and yield. They also suggested that efficacy of *Trichoderma* for plant growth promotion may be due to higher nutrient up take particularly N, P, K, Fe, Zn, and Cu by the crop. Similarly, Akgul and Mirik (2008) reported that the pepper treated with *Bacillus* strains M 1-3 and M 3-1 and their combination promoted plant growth and significantly reduced disease severity. Benitez *et al.* (2004) observed the production of organic acids, such as gluconic and citric acids that decrease the soil pH and permit the solubilization of micronutrients

and mineral cations like iron, manganese and magnesium, useful for plant metabolism.

Six PGPM and three antagonists selected after *in planta* screening were further identified based on their cultural and morphological characters. For species level identification of bacteria and actinomycetes 16S rDNA sequence analysis were also carried out.

Cultural and morphological characteristics of selected six bacterial isolates were studied on their respective media. These were further identified by 16S rDNA sequence analysis. The test isolates were identified by the accession in NCBI database, showing maximum homology with query sequence. The isolates YPTN-3 and HVKN-6 were identified as *Paenibacillus* sp., HPLPSB3 as *Acinetobacter grimontii*, HPLBC-6 as *Providencia* sp., HABBC3 as *Providencia* sp. and HPLPSB6 as *Paenibacillus polymyxa.*

Cultural and morphological characters of the selected best actinomycete HVZACT-1 was studied in detail on Kenknight's agar medium and based on that it was identified as *Streptomyces* sp. Further it was identified by 16S rDNA analysis confirmed as *Streptomyces termitum.*

Based on the cultural characters, best fungal antagonist FPRF-3 was identified as *Trichoderma* sp. Further NCFT identification was confirmed that the isolate FPRF3 as *Trichoderma viride*. The isolate HPLF-5 was then identified as *Gongoronella butleri.* Best antagonist which recorded least incidence and severity of foot rot disease was the *Trichoderma viride* (FPRF-3). In addition to this, it also improved the growth parameters such as length of vine, number of leaves and roots.

The present investigation was helpful in assessing the diversity of beneficial microflora in the rhizosphere of healthy and diseased black pepper plants in Wayanad district of Kerala State. Generally, population of microflora was more in rhizosphere

soil from healthy gardens. Bacteria, fungi, actinomycets, N_2-fixers, phosphate solubilizers, fluorescent pseudomonads and *Bacillus* were isolated on 15 different media. A total of 207 isolates were purified and further tested for antagonistic activities against the soil-borne plant pathogen *Phytophthora capsici.* Six most potential isolates after *in vitro* screening were selected to study their plant growth promoting activities in black pepper nursery. *Acinetobacter grimontii*, an efficient phosphate solubilizer and IAA producer was capable of promoting the length of vine, number of leaves and fresh root weight of seedlings. An isolate of *Providencia* sp., which produced maximum amount of IAA when tested under *in vitro* conditions, enhanced the root length of seedlings.

Three most potential PGPM isolates and three potential antagonists were evaluated *in planta*, against *Phytophthora capsici*, under artificially inoculated condition. Native isolate of the antagonistic fungus *Trichoderma viride* was not only capable of inhibiting the pathogen but also promoted the growth of plants. The actinomycete *Streptomyces termitum* was also efficient in suppressing the fungal pathogen. This is the first report of this actinomycete showing antagonistic activities against the dreadful fungal pathogen *Phytophthora capsici.* These isolates may further be evaluated under field conditions for plant growth promotion and disease suppression and may be exploited for biological control of foot rot disease in black pepper. The compatibility of the fungus *Trichoderma viride* and the actinomycete *Streptomyces termitum* may also be assessed so that a consortium of these two could be recommended for biological control of foot rot disease of black pepper. In addition to this, the compatibility of individual isolate and the consortium with chemical fungicides may also be assessed.

Summary

6. SUMMARY

The study entitled "Functional diversity of beneficial microorganisms from the rhizosphere of black pepper in Wayanad" was carried out in the Department of Agricultural Microbiology, College of Horticulture, Vellanikkara during 2012-2014. The major objective of the study was to assess the functional diversity of beneficial microorganisms in the rhizosphere of black pepper in Wayanad district with respect to plant growth promotion and antagonistic activities. Black pepper variety Panniyur-1 was used for the *in planta* experiment. The important findings of the study are summarized below:

- Twelve rhizosphere soils comprising four each from healthy, foot-rot affected and yellowing affected gardens were collected from black pepper gardens of Wayanad district.

- Population of different groups of microorganisms such as bacteria, fungi, actinomycetes, N_2 fixers, phosphate solubilisers, fluorescent pseudomonads and *Bacilli* were enumerated on 15 different media. Generally, population of microflora was more in rhizosphere soil from healthy gardens.

- A total of 207 isolates were purified, maintained and further tested for PGP and antagonistic activities against soil borne plant pathogen *Phytophthora capsici.*

- The isolate HPLBC-6 (*Providencia* sp.) recorded high IAA production of 292.50 $\mu g\ ml^{-1}$ in the presence of L-tryptophan.

- Quantitative estimation of solubilized P by the phosphomolybidic blue colour method revealed that HPLPSB-3 (*Acinetobacter grimontii*) was the most efficient one with 162.7 μg ml^{-1} in period of two weeks.

- The isolate YPTN-3 (*Paenibacillus* sp.) which recorded excellent growth on nitrogen free agar medium fixed maximum amount of nitrogen (46.92 mg) per gram of sucrose source provided in the medium.

- Six most potential PGPM isolates after screening were selected to study their plant growth promoting activities in black pepper nursery.

- *Acinetobacter grimontii*, an efficient phosphate solubilizer was capable of promoting the length of vine, number of leaves and fresh root weight of the cuttings.

- Three most efficient PGPM selected from *in planta* screening for growth promotion and three most promising antagonists selected based on *in vitro* antagonistic activity were evaluated *in planta.*

- Disease incidence was recorded minimum (6.23%) in T_6 (FPRF-3; *Trichoderma viride*) and this was followed by T_5 (HVZACT-1; *Streptomyces termitum*) with 13.20%. In case of per cent disease severity (PDI) also, least severity (4.00 %) was observed in T_6 (FPRF-3; *Trichoderma viride*).

- The isolate FPRF-3 had recorded production of NH_3 and siderophore under *in vitro* experiments. The same isolate had also recorded 100 per cent inhibition of the fungal pathogen when the culture filtrate was incorporated in the medium, indicating the presence of non-volatile metabolites capable of inhibiting the pathogen.

- Further intermingling, coiling and lysis of *P. capsici* by the antagonist FPRF-3 was confirmed microscopically.

- Cultural and morphological characteristics of selected isolates were studied on their respective media.

- The selected bacteria and actinomycete were further identified by 16S rDNA sequence analysis. The test isolates were identified by the accession in NCBI database, showing maximum homology with query sequence.

- The isolates YPTN-3 and HVKN-6 were identified as *Paenibacillus* sp., HPLPSB3 as *Acinetobacter grimontii*, HPLBC-6 as *Providencia* sp., HABBC3 as *Providencia* sp., HPLPSB6 as *Paenibacillus polymyxa* and HVZACT-1 as *Streptomyces termitum*.

- Based on the cultural characters, best fungal antagonist FPRF-3 was identified as *Trichoderma* sp. Further NCFT identification was confirmed that the isolate FPRF3 as *Trichoderma viride*.

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Appendices

APPENDIX I

MEDIA USED AND COMPOSITION

a) Ashby's N free agar

Mannitol	20.00 g
Dipotassium phosphate	0.20 g
Magnesium sulphate	0.20 g
Sodium chloride	0.20 g
Potassium sulphate	0.10 g
Calcium carbonate	5.00 g
Agar	20.00 g
Distilled water	1000 ml
pH	7.4±0.2

b) Czapek dox broth

Sucrose	30.00 g
Dipotassium phosphate	1.00 g
Potasium chloride	0.50g
Sodium nitrate	3.00 g
Magnesium suphate	0.50 g
Ferrous sulphate	0.01 g
Agar	20.00 g
Distilled water	1000ml
pH	7.3±0.2

c) Carrot agar

Carrot intrusions	20.00 g
Dextrose	20.00 g
Agar	20.00 g
Distilled water	1000 ml
pH	5.1

d) Jensen's agar

Sucrose	20.00 g
Dipotassium phosphate	1.00 g
Magnesium sulphate	0.50 g
Sodium chloride	0.50 g
Ferrous sulphate	0.10 g
Sodium molybdate	0.005 g
Calcium carbonate	2.00 g
Agar	20.00 g
Distilled water	1000 ml

e) Kenknight & Munaiers agar

Dextrose	1.00 g
Monopotassium dihydrogen phosphate	0.10 g
Sodium nitrate	0.10 g
Potassium chloride	0.10 g
Magnesium sulphate	0.10 g
Agar	20.00 g
Ditilled water	1000 ml

f) King's medium B Base

Proteose peptone	20.00 g
Dipotassium hydrogen phosphate	1.50 g
Magnesium sulphate. heptahydrate	1.50 g
Agar	20.00 g
Glycerol	15 ml
Distilled water	1000 ml
pH	7.2±0.2

g) King's medium A Base

Meat peptone	10.00 g
Casein peptone	10.00 g
Dipotassium phosphate	1.50 g
Magnesium sulfate	1.50 g
Agar	20.00 g
Glycerol	15.00 ml
Distilled water	1000 ml
pH	7.2 ± 0.2

h) Kuster's agar

Glycerol	10.00 g
Casein	3.00 g
Potassium nitrate	2.00 g
Sodium chloride	2.00 g
Dipotassium hydrogen phosphate	2.00 g
Magnesium sulphate	0.05 g
Calcium carbonate	0.02 g
Ferrous sulphate	0.01 g
Agar	20.00g
Distilled water	1000 ml

i) Luria Bertani broth (LB)

Tryptone	10.00 g
Yeast extract	5.00 g
NaCl	10.00 g
pH	7.50 g
Distilled water	1000 ml

j) Luria Bertani agar (LBA)

Tryptone	10.00 g
Yeast extract	5.00 g
NaCl	10.00 g
Agar	20.00 g
Distilled water	1000 ml
pH	7.2

k) Martin rose Bengal agar

Papaic digest of soyabean meal	5.00 g
Dextrose	10.00 g
Monopotassium phosphate	1.00 g
Magnesium sulphate	0.50 g
Rose Bengal	0.05 g
Agar	20.00 g
Distilled water	1000 ml
pH	7.2±0.2

l) Nutrient agar

Beef extract	3.00 g
Peptone	5.00 g
NaCl	5.00 g
Agar	20.00g
Distilled water	1000 ml

m) Nutrient broth

Beef extract	3.00 g
Peptone	5.00 g
NaCl	5.00 g
Distilled water	1000 ml

n) Pikovskaya's agar

Glucose	10.00 g
$Ca_3(PO_4)_2$	5.00 g
$(NH_4)_2SO_4$	0.50 g
NaCl	0.20 g
$MgSO_4.\ 7H_2O$	0.10 g
KCl	0.20 g
Yeast extract	0.50 g
$MnSO_4.H_2O$	0.002g
$FeSO_4.7H_2O$	0.002g
Distilled water	1000 ml
pH	7.0

o) **Potato dextrose agar**

Potato infusion	200.00 g
Glucose	20.00 g
Agar	20.00 g
Distilled water	1000 ml
pH	5.1

p) **Soil extract agar**

Soil extract	100 ml
K_2HPO_4	0.50 g
Glucose	1.00 g
Agar	20.00 g
Distilled water	1000 ml

APPENDIX II

REAGENTS USED

a) Ammonium molybdate reagent

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

a) Boric acid-indicator mixture

4% boric acid solution in hot water

b) Mixed indicator solution

0.2% bromocresol green + 0.2% methyl red in alcohol in 5:1 ratio

c) Picric acid solution

2.5 g of picric acid

12.5 g of Na_2CO_3

1000 ml of distilled water

d) Salkowski reagent

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

FUNCTIONAL DIVERSITY OF BENEFICIAL MICROORGANISMS FROM THE RHIZOSPHERE OF BLACK PEPPER IN WAYANAD

By

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THESIS

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ABSTRACT

Black pepper (*Piper nigrum* L.) is a perennial, woody and flowering climber belonging to family Piperaceae. It is one of the important spice crops which provides major source of income and employment for rural households in Kerala. Wayanad dominated in pepper farming in the state about 20 years ago. Annual production of pepper was 40,000 tonnes in the mid-1980s, which comprised about half of India's total pepper production. But recently, the production has declined drastically due to the infestation of pests and diseases. Foot rot caused by *Phytophthora capsici* and yellowing of black pepper are the major diseases devastating most of the plantations in Wayanad. However, some of the plants in the disease affected areas remain healthy which could be due to inherent activity of native rhizosphere microflora. The present study focused on assessing the functional diversity of beneficial microorganisms which could possibly be exploited for the benefit of plant growth.

Four healthy gardens, four gardens each affected by foot rot and yellowing were selected for sample collection. Rhizosphere soil samples were collected from five healthy vines in each garden. Population of beneficial microbes in the rhizosphere soils of healthy and disease affected gardens were compared. In general, rhizosphere soil from healthy gardens recorded higher population of bacteria, fungi, phosphate solubilizers and fluorescent pseudomonads. A total of 207 isolates (including 112 bacteria, 32 actinomycetes and 63 fungi) were purified and maintained to study their plant growth promoting and antagonistic activities.

Maximum IAA production (292.50 $\mu g\ ml^{-1}$) was recorded by HPLBC-6 followed by HABC-3 (46.43 $\mu g\ ml^{-1}$). The isolate HPLPSB-3 was the most efficient P solubiliser (162.7 $\mu g\ ml^{-1}$) followed by HPLF-5 (161.3 $\mu g\ ml^{-1}$). The isolate YPTN-3 fixed maximum amount of nitrogen (46.92 mg of N g^{-1} of sucrose) followed by HVKN-6 (32.62 mg of N g^{-1} of sucrose).

From the *in vitro* experiment, two most promising isolates each of IAA producers, phosphate solubilizers and nitrogen fixers were selected for preliminary screening for growth promotion on black pepper cuttings. The isolate HPLPSB-3 (P solubiliser) recorded maximum sprouting, vine length, number of leaves, number of roots and roots fresh weight under *in planta* screening for plant growth promotion. However, maximum root length was observed in HPLBC-6 (IAA producer).

All the isolates were screened *in vitro* for their antagonistic activity against foot rot pathogen *Phytophthora capsici*. Among the bacteria, isolate HPLPSB-6 recorded maximum inhibition (69.27 %) of the pathogen. Among the actinomycetes, HVZACT-1 recorded maximum mycelial inhibiton of 66.66 %. Among the fungal isolates screened, maximum inhibition (75.17 %) was recorded by the isolate FPRF-3.

The three most promising PGPM selected from preliminary *in planta* screening and three antagonists from *in vitro* screening were further tested for their efficiency in controlling foot rot disease in black pepper nursery. Minimum disease incidence (6.23%) and severity (4.00 %) were observed in isolate FPRF-3. This was followed by actinomycete HVZACT-1 with disease incidence of 13.20 % and severity of 8.00 %. Maximum disease incidence and severity were observed in control with pathogen alone. In addition to biocontrol activity, FPRF-3 also improved plant growth parameters such as length of vine, number of leaves and roots.

The selected growth promoting isolates HPLPSB-3, HPLBC-6 and YPTN-3 were identified as *Acinetobacter grimontii, Providencia* sp. and *Paenibacillus* sp. The three selected antagonists HPLPSB-6, HVZACT-1 and FPRF-3 were identified as *Paenibacillus polymyxa*, *Streptomyces termitum* and *Trichoderma viride* respectively.

Based on *in planta* evaluation, *Acinetobacter grimontii* was considered as the best PGPM and *Trichoderma viride* the most promosing antagonist against *P. capsici*. These isolates could be further exploited for improving the growth and managing foot rot disease, after validation under field conditions. The compatibility of PGPM with antagonists and chemical fungicides may also be evaluated. This is the first report of antagonistic activity of the actinomycete *S. termitum* against *P. capsici* causing foot rot disease in black pepper.