PLANT GROWTH PROMOTION AND FOOT ROT DISEASE SUPPRESSION IN BLACK PEPPER USING FUNGAL AND BACTERIAL ENDOPHYTES

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by

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(2018-11-092)

THESIS

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2020

DECLARATION

I, hereby declare that this thesis entitled "**PLANT GROWTH PROMOTION AND FOOT ROT DISEASE SUPPRESSION IN BLACK PEPPER USING FUNGAL AND BACTERIAL ENDOPHYTES***"* is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis entitled "**PLANT GROWTH PROMOTION AND FOOT ROT DISEASE SUPPRESSION IN BLACK PEPPER USING FUNGAL AND BACTERIAL ENDOPHYTES"** is a record of research work done independently by **Ms. TEENU PAUL (2018-11-092)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

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"Coming together is a beginning, Keeping together is progress,
Working together is success"

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Teenu Paul

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LIST OF ABBREVIATIONS AND SYMBOLS USED

et. al.	And other co-workers
Cfu	Colony forming unit
Cm	Centimetre
CRD	Completely randomized design
CD	Critical difference
DAI	Days after inoculation
DAT	Days after transplanting
^{0}C	Degree celsius
DNA	Deoxyribo nucleic acid
Fig.	Figure
FAO	Food and Agricultural Organisation
G	Gram
H	Hours
Ha	Hectare
IAA	Indole Acetic Acid
M	Metre
Mg	Microgram
µL	Microliter
Mg	Milligram
viz.,	Namely
Nm	Nanomertre
NS	Non-significant
No.	Number
NA	Nutrient agar
NB	Nutrient broth
OD	Optical Density
%	Per cent

PGPR	Plant growth promoting rhizobacteria
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
Rpm	Rotations per minute
sp. or spp.	Species (singular and plural)
SE (m)	Standerd error (Mean)
var.	Variety
w/v	Weight by Volume

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Introduction

1. INTRODUCTION

Black pepper (*Piper nigrum* L.) is a flowering vine that belongs to family Piperaceae. It is one of the major spice crops which has its origin in Western Ghats region in South India. This spice is mainly cultivated in Guatemala, Indonesia, Brazil, Thailand, Malaysia, China, India, Sri Lanka, Cambodia, Mexico, and Vietnam. Black pepper cultivation in India is mainly limited to Kerala and Karnataka. States like Tamil Nadu, Andhra Pradesh, Maharashtra, and north eastern states also produce black pepper (Shashidhara, 2007).

Major portion in the Indian spice scenario is contributed by black pepper. It is the "King" among spices. It is an important export-oriented spice crop valued for its berries both in the white and black forms and also for its oleoresins, essential oil and other value-added products. Indian pepper is having high demand in International market since ancient ages. Black pepper has wide use apart from human dietaries. It is a major component in most of ayurvedic medicines, used as preservative in food industry, essential oils from pepper is used in perfumery and also be used as an insecticide

Black pepper plants are affected by pathogen *Phytophthora capsici* which is the causal organism of foot rot diseases. This is one among the major constraints faced by black pepper growers of Kerala, especially during the southwest monsoon season (Sarma *et al*., 1994). This disease develops during monsoon season where conditions like well distributed rainfall of about 2,500 mm along with shorter sunshine duration for one to three hours. The beginning of epiphytotics of *Phytophthora* diseases is contributed by soil borne inoculum (Thorold, 1955; Onesirosan, 1971). Water and soil act as agents of spread of this disease (Nambiar and Sarma, 1977). Black pepper vine is susceptible to this pathogen at any of its development stage. This pathogen attacks all the above and below ground parts of the vine which includes root, collar, leaves, stem, berries and inflorescence. The recommended practices for the management of this deadly disease is possible only through phyto sanitary measures and repeated preventive spraying or drenching of vines with of copper fungicides. Bioagents have been used for foot rot

disease management and plant growth promotion in black pepper. They include rhizospheric and endophytic bacteria and fungi.

The increased use of hazardous fungicides in the field of agriculture in recent years has been subject to discussion of conservationists and public health workers. Use of microbes which can be utilized as plant health managers is a safe alternative, as this method demands low amounts of chemical inputs to farming systems, which in turn results in reduction in cost of pest and disease control measures. This also reduces pollution hazards, with less disturbances of biological equilibrium (Senthilkumar *et al.*, 2011).

Endophytic bacteria can be defined as a class of endosymbiotic microorganisms that usually colonizes in the internal tissues of host plants without causing any injuries (Schulz and Boyle, 2006). Endophytes are involved in diverse roles in plant health such as in the disease resistance, nitrogen fixation, solubilization of immobilized phosphorus, nutrient cycling, stress tolerance and production of plant hormones, siderophores and volatile organic compounds (Sturz *et al*., 2000). The bacterial endophytes *Bacillus velezensis* PCSE10 and *Rhizobium radiobacter* PCRE10 isolated from the exotic pepper variety *Piper colubrinum* had plant growth promotional activity in black pepper plantlets and biocontrol activity against *Phytophthora capsici* (Kollakkodan, 2017; Kollakkodan *et al*., 2017).

Piriformospora indica, belongs to the order Sebacinales in Basidiomycota is a biotrophic mutualistic root endosymbiont (Verma *et al.*, 1998; Varma *et al*. 1999; Weiss *et al.*, 2004). It is an axenically cultivable root endophyte which imitates the abilities of arbuscular mycorrhizal fungi (AMF). *P. indica* is having wide host range including bryophytes, pteridophytes, gymnosperms and angiosperms (Shahollari *et al*., 2005; Deshmukh *et al*., 2006). Symbiotic association of *P. indica* induces abiotic stress tolerance against salinity, drought, heavy metal toxicity, and low temperature (Baltruschat *et al*., 2008; Sheramati *et al*., 2008; Zarea *et al.,* 2012). This fungus improves defensive capacity of several crop plants against bacterial, fungal, nematode

and viral pathogens (Lakshmipriya *et al*., 2017; Athira, 2018; Ashar, 2019; Chandan, 2019). *P. indica* can also acts as a potential biological hardening agent for micro propagated plantlets (Li *et al*., 2019).

Bio inoculation with a combination of the endophytic fungus *Piriformospora indica* and the endophytic bacterial strains is expected to have beneficial effect on growth of bush pepper and foot rot disease suppression in black pepper.

Hence the present programme has been undertaken with major thrust on the following aspects.

1. To assess and evaluate the compatibility of the root endophytic fungus *Piriformospora indica* and two endophytic bacterial strains *Bacillus velezensis* PCSE 10 and *Rhizobium radiobacter* PCRE 10
2. To assess effect of the root endophytic fungus *Piriformospora indica*, two endophytic bacterial strains and their combination on growth promotion in bush pepper and foot rot disease suppression in black pepper.

Review of literature

2. REVIEW OF LITERATURE

Black pepper is a perennial climbing plant *Piper nigrum* L. which belongs to the family Piperaceae (Ravindran, 2000). Major portion in the Indian spice scenario is contributed by black pepper. It is the "King" among spices. It is one of the oldest spices known to the world which has its origin in the humid, tropical evergreen forests of South West India, particularly the Western Ghats regions of the South India (Ravindran, 2003). This spice was once produced only in the western coastal region of India, from where its cultivation spread to most tropical countries and to their colonial countries by the efforts of European countries wherever a favourable climate for growth and production existed (Ahmad *et al.,* 2012). First oriental spice to be introduced into the Western world was black pepper, and it was well known among the Greeks and Romans. Pepper assumed considerable demand in Europe in the medieval periods. Introduction and use of black pepper resulted in complete changes in western cooking (Ravindran and Kallupurackal, 2012).

Black pepper cultivation in India is mainly limited to Kerala and Karnataka. States like Tamil Nadu, Andhra Pradesh, Maharashtra, and north eastern states also produce black pepper. *Piper nigrum* has various common names, viz. Kali mirch in Hindi, Kala morich and gol morich in Bengali, Kala mari and kalo mirich in Gujarati, Kare menasu in Kannada, Marutis in Kashmiri, Kurumulaku in Malayalam, Mira and kali mirch in Marathi, Gol maricha in Oriya, Kali mirch in Punjabi, Marichushna and hapusha in Sanskrit, Milagu in Tamil, Miriyalalu in Telungu, Kali mirch and Siah mirch in Urdu. Apart from India, pepper is now cultivated in Brazil, Cambodia, China, Guatemala, Indonesia, Malaysia, Mexico, Srilanka, Thailand, and Veitnam.

Vietnam, Brazil, Indonesia, India, Malaysia and Sri Lanka are the major contributors to international markets. The share of India to the world market is around 10% (Krishnamoorthy and Parthasarathy, 2009). Pepper accounts for about 34 % of total spices traded globally (Ravindran and Kallupurackal, 2012).

Black pepper plant reaches heights of 33 feet (10 meters) by help of its aerial roots. It is woody climber with alternately arranged leaves. The flowers are in the spikes with 50 blossoms. Fruits are drupes of around 0.2 inch (5 mm) in diameter, which are known as peppercorns in market. Fruits at maturity changes to yellowish red from its green colour and carry a one seed. Black pepper is a vine which is commercially raised from orthotropic stem cuttings. It requires a tree or pole for support. Black pepper is a self-pollinated crop. Areas receiving long rainy seasons, with partial shade and high temperature are favourable for its growth. Berries starts harvesting from three to five years. It continues to yield for about forty years.

Black pepper has wide use apart from human dietaries. It is a major component in most of ayurvedic medicines, used as preservative in food industry, essential oils from pepper is used in perfumery and also be used as an insecticide (Srinivasan, 2007). Oleoresin, the essential oil responsible for the aroma of pepper and the alkaloid piperine contributing to the pungency (Srinivasan, 2007).Essential oil from black pepper is the oleoresin and the plant alkaloid which contributes to the unique pungency is the Piperine have many advantages which make pepper been preferred in most of the processed foods are its ease of commercial handling and absence of microbial contamination and decay

Black pepper has many versatile biological applications. This includes treatment of asthma, fever, obesity, chronic indigestion, vertigo, cholera, congestion, and arthritic disorders (Alodeani *et al*., 2015).

The productivity of black pepper is slowly declining, mainly because of the prevalence of epidemic diseases, pests and drought. The increased use of chemical pesticides leads to contamination of produce as well as environment with residues of pesticides. ‘Clean spices’ and organic spices are the concepts that are catching on and this is achieved through integrated approaches for pest, disease and nutrient management involving resistant varieties, biocontrol, botanicals and organic farming. This also reduces soil degradation.

2.1 BUSH PEPPER

Bush pepper plants is a miniature form of black pepper with limited upward growth which are raised from plagiotrops (lateral branches) which bears fruits on black pepper. Bush pepper can grow as a garden plants without the need for standards for support. Nowadays, there is an increase in demand for bush pepper planting materials (Mol *et al.,* 2017). One can harvest spikes without the help of climbers at any season. Perennial vine, black pepper is having a long juvenile period of about 3-4 years before reproductive phase. Whereas, bush pepper plants start bearing fruits in the same year of planting. Flowering can be obtained throughout the year, when the plants are maintained with irrigation continuously.

Bush pepper is easy to maintain in land-limiting situation such as apartments and in non-traditional areas where black pepper is not grown. They can be grown in larger pots or in fields at a spacing of 2m×2m and continue flowering during all season if manured and watered regularly (IISR, 2002). Use of bush pepper system as a model system for estimating requirements for black pepper plants. This includes requirement of light (Devadas and Chandini, 2000), nutrients (Hamza and Sadanandan, 2005), soil (Thankamani and Ashokan, 2007), water (Srinivasan *et al.,* 2013) for *Piper nigrum*. Anith *et al.* (2018) conducted an experiment to assess the impact of root colonization by *P. indica*, an endophytic fungus. The study revealed a significant increase on growth and characteristics like piperine and essential oil content in bush pepper.

Rooting of plagiotrops was found to be less as compared to runner shoots. One of the major steps in the production of bush pepper is standardization of suitable rooting conditions. Mol *et al.* (2017) conducted a study to investigate the rooting and establishment of laterals. Coir pith compost was incorporated in the potting mixture and the plants were kept under different humidity conditions. When plagiotropic branches of pepper variety, Panniyur - 1 were planted in polyethylene bags and maintained under 50% shade, a success rate was found 35percent (Lakshmana *et al.,* 2016). On a study

conducted in one of the farmer's nursery, percentage of establishment of bush pepper was found varied from 10-40% (Prakash *et al.,* 2016).

2.2 PATHOGEN *Phytophthora capsici*

Foot rot disease, a major disease in black pepper caused by the fungal pathogen *Phytophthora capsici*. Leon H. Leonian was credited for describing *P. capsici* for first time in 1922 at the New Mexico Agricultural Research station in Las Cruces. This is a soil borne fungus and all parts at any stage of black pepper is susceptible to this pathogen (Sarma *et al.,* 1994; Anandaraj and Sarma, 1995). Screening of most of the available germplasm is carried out against this pathogen and it is found that no cultivated variety is found resistant and there is no genetic basis for disease resistance (Krishnamoorthy and Parthasarathy, 2009).

Black pepper plants at any of its growth stages are affected by pathogen *Phytophthora capsici* which is the causal organism of foot rot diseases. This is one among the major constraints faced by black pepper growers of Kerala, especially during the southwest monsoon season (Sarma *et al*., 1994). This disease develops during monsoon season with well distributed rainfall of about 2,500 mm with shorter sunshine duration for one to three hours. Maximum disease development was noticed when temperature range 18.5-22.5^{0}C coupled with 95 percent relative humidity (Shamaro and Sidharamiah, 2002).

The beginning of epiphytotics of *Phytophthora* diseases is contributed by soil borne inoculum (Thorold, 1955; Onesirosan, 1971). Water and soil acts agents for mode of spread of this pathogen (Nambiar and Sarma, 1977). Black pepper vine is susceptible to this pathogen at any of its development stage. This pathogen attacks all the above and below ground parts of the vine which includes root, collar, leaves, stem, berries and inflorescence. The recommended practices for the management of this deadly disease is possible only through phyto sanitary measures and repeated preventive spraying or drenching of vines with of copper fungicides.

2.2.1 Biological control of *P. capsici*

The increased use of hazardous fungicides in the field of agriculture in recent years has been subject to discussion of conservationists and public health workers. Use of microbes which can be utilized as plant health managers is a safe alternative, as this method demands low amounts of chemical inputs to farming systems, which in turn results in reduction in cost of pest and disease control measures. This also reduces pollution hazards, with less disturbances of biological equilibrium (Senthilkumar *et al.*, 2011).

Traditionally the management of nursery diseases are by use of chemicals, but the continuous application of such chemicals cause deterioration in the quality of environment by polluting the water, soil and other natural resources. An alternative to such chemicals is eco-friendly techniques which include use of resistant varieties, sanitary and phytosanitary (SPS) measures coupled with biological control measures. Till now, no resistant varieties have been reported showing resistance against foot rot pathogen (Shashidhara, 2007). Success of eco-friendly measures especially biological control involves multitude of factors such as varietal, soil, agronomical, and environmental factors.

One of the emerging areas in management of plant pathogens in recent times is biological control (Whipps, 1997; Bowen and Rovira, 1999). Several studies showed the use of *Pseudomonas* spp. as a biocontrol agent in various crops was found effective in controlling soil borne plant pathogens. (Weller, 1988).

Cristinzio (1987) studied antagonistic property of *Trichoderma* and *Chaetomium* spp. against *P. capsici* under *in vitro* conditions. A study conducted by Sodsa-art and Soytong (1999) reported that *Trichoderma* and *Chaetomium* mycofungicides when applied as mixture significantly reduced root and stem rot disease of black pepper caused by *Phytophthora palmivora.* The lowest disease incidence was obtained was 8.6 per cent, when mycofungicides applies as mixture. 10.9 percent disease incidence was observed in

Trichoderma applied plants followed by 22.6 percent in plants applied with *Chaetomium* alone. All treatments showed significantly lower disease incidence than the non-treated ones with the highest disease incidence of 71 per cent.

Anandaraj and Sarma (1995) reported biocontrol agents against the *Phytophthora* foot rot of pepper. This study used *Glomus virens, Glomus fasciculatum,* and *T. hamatum*. Bacterial antagonists can be utilized for biological control of foot rot disease (Jubina and Girija, 1998). A study conducted by Anith and Manomohandas (2001) revealed that *Phytophthora capsici* induced nursery rot incidence can be reduced by the application of *Trichoderma harzianum* and *Alcaligenes* sp. Strain AMB 8 alone or in combination. Biological control by antagonistic bacteria was found possible in nursery wilt (Anith *et al*., 2003). An *in vitro* evaluation conducted by Anandaraj and Sarma (2003) revealed the per cent inhibition in the growth of *Phytophthora capsici* varied between 0 to 84 per cent by using different isolates of *Trichoderma* spp., whereas bacterial isolates inhibited *P. capsici* upto 50 per cent.

Plant pathogens can be controlled biologically by the application of endophytic microorganisms (Hallmann *et al*., 1997; Kobayashi and Palumbo, 2000; Sturz *et al*., 2000; Ryan *et al*., 2008). Aravind *et al.* (2009) reported the endophytic bacteria isolated ie, *Bacillus* and *Pseudomonas* bacteria effectively control foot rot disease in black pepper caused by *Phytophthora capsici*.

2.3 ENDOPHYTES-A NOVEL TOOL FOR PLANT GROWTH PROMOTION AND DISEASE SUPPRESSION

The productivity of farming systems has been improved by the use of pesticides and fertilizers which are used for controlling pests and supplying nutrients for growth of plants. Hazell and Wood (2008) reported the negative effects of long-term use of chemical products in field of agriculture which negatively affect the soil quality by reducing the yielding capacity, pollutes the underground water and destroys biodiversity. Also, the continuous use of fertilizers affects public health. Nowadays there is growing

concern among public regarding the after effects of long-term use of agrochemicals. High input agriculture production methods and indiscriminate use of pesticides and fertilizers resulted in decline in plant resistance to abiotic and biotic stress factors. Use of microorganisms which benefits plants contributes positively to sustainable agriculture is an environmentally safe alternative for agrochemicals (Vessey, 2003). The application of bioagents are helpful in agriculture. This includes growth promotion, disease control, yield increases, and quality upgradation. Studies are conducted in recent years to developed safe choices to fertilizers and pesticides ie, formulated products of biofertilizers and biocontrol agents (Gray and Smith, 2005; Singh *et al*., 2011). Microbial inoculants with beneficial effects used in agriculture are bacteria and fungi with growth promoting effects. According to their functions they are grouped in biofertilizers and biocontrol agents. According to Malusa and Vassilev (2014), biofertilizers and biocontrol agents are products formulated with one or more microorganisms that improve the level of essential nutrients in soil and health of the crops. This is possible either by substituting soil nutrients or by making nutrients more accessible to plants or by synthesising specific metabolites.

The term endophyte was introduced by De Bary in 1866. The word endophyte is a combination of two Greek words, *endon* and *phyton* which means within and plant respectively. Freeman in 1904 is credited with isolating first endophyte from *Lolium temulentum* (Kusari *et al*., 2012).

Endophytes are defined as microbes that colonize internal tissues of living plants without causing any immediate, overt negative effects (Bacon and White, 2000).

Endophytes are having many beneficial effects to plants. They help in nitrogen fixation (Dobereiner *et al.,* 1993), pathogenic resistance (Chen *et al.,* 1995; Backman *et al*., 1997), stress tolerance (Sturz and Nowak, 2000), solubilization of immobilized phosphorus (Guo *et al*., 2000), in the production of plant hormones (Lee *et al.,* 2004), synthesis of siderophore (Prasad and Dagar, 2014) and production of volatile organic compounds (Sheoran *et al.,* 2015).

Several diazotrophic endophytes which can provide fixed Nitrogen have been isolated from rice (Gyaneshwar *et al.,* 2001; 2002; Verma *et al.,* 2001). Kobayashi and Palumbo (2000) stated that endophytic bacteria are of increasing interest due to their potential uses in agriculture. Ryan *et al*. (2008) has reviewed applications of bacterial endophytes in sustainable agriculture. Exploitation of plant- endophyte interactions can result in the plant health promotion and also plays a major role in sustainable agriculture for all crops by decreasing health problems and environmental pollution caused by the indiscriminate use of agrochemicals and Nitrogen fertilizers to obtain higher production.

Bacillus and *Pseudomonas* are the genera of endophytic bacteria which are commonly encountered in most of agricultural crops (Seghers *et al.,* 2004; Aravind *et al.,* 2009: Souza *et al.,* 2013). Kollakkodan *et al*. (2017) isolated different bacterial endophytes from both *P. colubrinum* and *P. nigrum.*

Rhizobium sp. as endophyte in the roots of rice plants were reported (Yanni *et al.,* 1997; Chi *et al.,* 2005). Presence of *Rhizobium* sp. as an endophyte in two species of *Piper* were reported. *viz, P. tuberculatum* and *P. colubrinum* (Nascimento *et al*., 2015; Kollakkodan *et al*., 2017).

Rhizobium radiobacter is a soil bacterium. This bacterium is well known for its ability to infect dicot plants and frequently results in crown gall disease. Plant tissues infected by crown this disease are distinctive by the uncontrolled division of cells which leads to increasd size of organs. Integration of a bacterial virulence plasmid (Ti) from bacterium into the plant genome results in tumour induction. which contains Genes that codes for phytohormones auxin and cytokinin are present in Ti plasmid.

Humphry *et al.* (2007) reported that increased root and shoot length in barley by *R. radiobacter* strain 204. It also improved grain yield in barley and wheat and this strain used in Russia on graminaceous crops as a biofertilizer. Sharma *et al.* (2008) reported *Rhizobium radiobacter* PABac-DSM when applied on barley seedlings of three days old

by dip inoculation induces growth promotion. In that experiment there was 17% increase over control plants in shoot fresh weight was noticed after three weeks.

Sharma *et al.* (2008) detected close association between *Rhizobium radiobacter* and *Piriformospora indica*. Sharma *et al.* (2008) have documented indole-3-acetic acid (IAA) production by *R. radiobacter* PABac-DSM after 24 h when inoculated in mineral salt medium supplemented with 0.5% glucose and 500 mg ml^{-1} of tryptophan at 25°C was found upto 40 mg/ml. Absence of IAA production was noticed when tryptophan is absent in medium of demonstrating *R. radiobacter* PABac-DSM follows tryptophan dependent IAA synthesis.

Induced systemic resistance (ISR) defined by Kloepper *et al*. (2014) as the process by which plants treated with PGPR leads to the elicitation of host defense visible by reduction in incidence of diseases caused by plant pathogens that are spatially separated from inducing agent.

Some strains of *Bacillus* spp. were found reducing the severity and incidence of viral diseases in green house conditions. A study conducted by Murphy *et al.* (2003) using potting mix incorporated with *Bacillus* spp., they found significant reduction in disease severity in all the bacterial treatments. They studied parameters like symptoms, disease incidence, and virus accumulation and found that there was reduction in all these parameters. The study also showed a significant increase in plant fresh weight, number of fruits and flowers in treated plants when challenge inoculation with CMV was not done. The bacterial treated plants showed higher level of protection and the plant growth promotion response and it is generally similar to ten days older untreated plants

Treatment with *Bacillus* spp. reduced the severity of blue mold of tobacco, caused by *Peronospora tabacina* (Zhang *et al.,* 2002a; 2002b; 2004). The results of their work showed an association between *Bacillus* spp. in growth promotion and ISR elicitation.

Greenhouse tests conducted by Zehnder *et al.* (1997) to determine effect of *B. pumilus* strain INR7 on Cucurbit wilt disease severity in cucumber plants. In this study,

seed treatments and drenching of bacteria were done. Transmission pathogen from infected to healthy plants was done with one of the vectors of the disease. There was significant reduction in number of wilted leaves on the plants treated with *B. pumilus* strain INR7 than without bacterial treatment.

Yan *et al*. (2003) demonstrated when potting medium was incorporated with *B. pumilus* strain SE34. Later, challenge-inoculation with *Phytophthora infestans* was done in all plants and it was found that disease severity significantly reduced on plants treated with *B. pumilus* strain SE34.

A significant increase in plant height and overall plant weight was obtained on tomato plants treated with *B. pumilus* strain SE34 compared with non-bacterized plants (Yan *et al.,* 2002). This strain was found to elicit ISR on tomato plants (Ryu *et al*., 2003: 2004).

As reported by several authors *Bacillus* is an ideal candidate for plant growth promotion and biocontrol of plant pathogens because of its ability to form endospores which are resting structures capable of surviving desiccation, heat, oxidising agents, UV and gamma radiations (Turner and Backman, 1991; Shafi *et al*., 2017).

These attributes endow *Bacillus* an ecological advantage over several other biocontrol bacteria and render it amenable to commercialization due to the extended shelf life. In addition, it also produces highly stable antibiotics that are detrimental to several pathogens (Baker and Cook, 1974; Handelsman *et al*., 1990; Osburn *et al*., 1995; Weller, 1988; Shafi *et al*., 2017).

2.3.1 Plant growth promoting traits of endophytic bacteria

Indole Acetic Acid (IAA) is considered to be the most important native auxin. IAA belongs to the group of phytohormones (Strzelczyk and Pokojska, 1984). Endophytic bacteria within the plant release auxin which results in the formation of

lateral, adventitious roots and root hairs thereby increasing the root surface area thus promote plant growth (Hilbert *et al*., 2012).

Yingwu *et al*. (2009) reported that some endophytic microorganisms upon colonization on plants increase growth which attributed to their potential to synthesize IAA. Tryptophan is used as the precursor by microbes in the biosynthesis of IAA via tryptophan dependent route. Bacterial diazotrophs like *Azotobacter, Serratia* etc. have reported to have the ability to synthesis IAA (Celloto *et al*., 2012). Bacterial IAA in conjugation with endogenous auxins produced by host plant stimulates promotion of growth.

Bianco *et al*. (2006) stated that IAA production by endophytic bacteria results in increase in host plant growth. Bacterial IAA can provide protection against stresses by improving different cellular defense systems. Several authors reported the detection of plant growth promoting beneficial microorganisms can be detected by screening based on IAA production (Ali and Hasnain, 2007; Govindarajan *et al*., 2007; Jasim *et al*.,2013).

Jasim *et al.* (2013) have screened seven bacterial isolates from *P. nigrum* based on IAA production. Prasad and Dagar (2014) reported IAA production by endophytes isolated from Avocado and grapes with maximum production by strain SA 3.

Ammonia production is a major trait of PGPR which effects plant growth indirectly. An amino acid tryptophan is hydrolyzed into indole, pyruvic acid, and ammonia by enzyme tryptophanase which is present in bacteria. Pyruvic acid and ammonia are used by bacteria to satisfy its nutritional needs (Prescott and Harley, 2002). Incorporation of ammonia nitrogen into organic material relatively easy and direct. Because as compared to other forms of inorganic nitrogen, ammonia nitrogen is more reduced. In the Rhizobium legume interaction, the ammonia in the bacterial cell diffuses out and is assimilated in the legume cell (Prescott and Harley, 2002).

2.3.2 Endophytic Bacteria as Biocontrol Agents

A diversity of endophytes reported with superior antagonistic potential against bacterial and fungal pathogens (Van Buren *et al.,* 1993; Chen *et al*., 1995; Sturz *et al*., 2000; Lodewyckx *et al.,* 2002; Reiter *et al.,* 2002). Protection of the plants from pathogens by bacterial endophytes are by production of antibiosis, out competition of pathogens and induced systemic resistance (Hallmann *et al*., 1997; Sturz *et al*., 1999; Sessitsch *et al*., 2004). ISR is a mechanism of biocontrol due to the intimate contact between plant and endophytes (Chen *et al.,* 1995; Benhamou *et al*., 1996; Hallmann *et al.,* 1997; Nejad and Johnson, 2000; Reiter *et al*., 2002).

The success of biological control of plant diseases by using endophytes is dependent on many factors. The factors include the host specificity, the population dynamics and pattern of host colonization. Also, the ability of endophytes to move within host tissues, and the ability to induce systemic resistance influences the biological control of pathogens by endophytic microorganism (Backman *et al*., 1997).

Mahaffee and Kloepper (1997) has reported the possibility of using endophytic bacteria as biological control agents. Endophytic bacterial provides induced resistance to soil borne pathogens. An experiment conducted by Chen *et al.* (1995) revealed the reduction in disease symptoms and incidence of *Fusarium oxysporum* in cotton on inoculation with some bacterial endophytes. Inhibition in the growth of *P. ultimum* and *F. oxysporum* under *in vitro* conditions was found possible by the antibiotics produced by *Streptomyces* sp. strain NRRL 30562 which was isolated from *Kennedia nigriscans* (Castillo *et al.,* 2002). Sessitsch *et al*. (2004) reported a reduction in the growth of *Streptomyces scabies* and *Xanthomonas campestris* on *in vitro* conditions is possible the endophytic bacteria isolated from potato plants. The growth of pathogen is inhibited by the antibiotics and siderophores produced by the endophytes.

Endophyte can produce antibiotics. *P. fluorescens* strain FPT 9601 was found to synthesize 2, 4-diacetylphloroglucinol (DAPG). DAPG crystals synthesized are deposited around tomato roots (Aino *et al.,* 1997).

ISR activity was also found in endophytic bacteria. Benhamou *et al.* (1996) observed ISR activity against *F. oxysporum* f. sp. *pisi* on roots of pea plants was triggered by *B. pumilus* SE34 Conn *et al.,* (1997) also observed ISR activity against *F. oxysporum* f. sp. *vasinfectum* on cotton roots by *B. pumilus* SE3. M'Piga *et al.* (1997) reported ISR was triggered by *P. fluorescens* against *F. oxysporum* f. sp. *radices-lycopersici* in tomato.

2.3.3 Endophytic bacteria from *Piper*

Benchimol *et al.* (2000) identified a *Methylobacterium radiotolerans* which was found reduced mortality of seedlings caused by root rot disease in black pepper. Anith *et al.* (2003) isolated bacterial antagonists from rooted cuttings of black pepper and found that most of the isolates showed different levels of antagonism against *P. capsici* in the dual culture and the shoot assay. By shoot assay, isolate PN-026 was found to be effective in reducing nursery wilt of black pepper and also its treatment showed increased shoot growth which indicates that this isolate is having growth promoting effect.

Aravind *et al.* (2009) identified diverse group of endophytic bacteria from black pepper having antagonistic effect against *P. capsici.* The study revealed three black pepper associated endophytic bacteria isolated were found promising for suppression of *P. capsici*. Pre plant bacterisation with these three endophytic bacteria resulted in production of disease free rooted black pepper plantlets by about more than 60% (Aravind *et al.,* 2012).

Piper colubrinum, a wild exotic relative of black pepper, is resistant to the foot rot pathogen. Kollakkodan *et al*. (2017) isolated the culturable bacterial endophytic communities associated with *Piper nigrum* and *Piper colubrinum* and screened for antagonistic potential against *Phytophthora capsici.* Out of the 47 bacterial endophytic isolates obtained, 12 showed *in vitro* antagonism against *P. capsici*. Phylogenetic analysis

of 16S rRNA gene sequences showed considerable diversity of antagonistic bacterial endophytes present in *Piper* spp. Kollakkodan *et al*. (2017) observed that *Bacillus* spp. were predominant among the endophytes that have antagonistic activity against *P. capsici*. An endophytic bacterial isolate belonging to *Rhizobium* sp that showed *in vitro* antagonism against *P. capsici* was obtained from *P. colubrinum* root tissues. Kollakkodan *et al*. (2017) reported that endophytic bacteria from *Piper colubrinum* had more antagonistic action against *P. capsici* than those from *P. nigrum* and also two strains that found to have antagonistic and growth promoting effects were *Bacillus velezensis* PCSE 10 and *Rhizobium radiobacter* PCRE 10.

Nascimento *et al.* (2015) identified endophytic bacteria associated with roots *Piper tuberculatum*, which is resistant to infection by *Fusarium solani* f. sp *piperis*, causing root rot disease in black pepper. Nascimento *et al.* (2015) identified 13 genera of endophytes isolated from *Piper tuberculatum.* Based on antagonistic assays they identified *Pseudomonas putida* and *Pseudomonas* sp, were found to inhibit *F. solani* f. sp *piperis* growth *in vitro*.

Jasim *et al*. (2013) isolated of twelve bacterial endophytes from black pepper which were later screened for various plant growth promotional properties like IAA production, phosphate solubilization, ACC deaminase production and siderophore production. They found seven isolates which have IAA biosynthetic potential. Also identified *Klebsiella* sp and *Enterobacter* sp. as the isolates with excellent growth promoting properties on *Vigna radiata* seedlings.

2.4 *Piriformospora indica* - A PLANT GROWTH PROMOTING ROOT ENDOPHYTIC FUNGUS

Mycorrhizal fungi and plant-growth promoting microorganisms like *Rhizobium*, Phosphorus solubilizing microbes etc. were found to play an important role in plant growth. They manifest various mechanisms in plants to obtain final result (Vassilev *et al.*, 2017). During last decades several developments took place in the field of development

of microbial formulations. *Piriformospora indica* which belonging to Basidiomycetes were found to produce some beneficial effects on plants. This includes growth promotion and Phosphorus solubilization. This fungus can grow in axenic conditions and are easy to handle. Mass multiplication of this fungus is possible in conventional fermentation systems. When culture filtrate obtained from *P. indica* was introduced into soil- water systems demonstrated with same effects as spores/mycelium (Bagde *et al*., 2011; Kumar *et al*., 2012).

Piriformospora indica ia an AM like fungus which belongs to the order Sebacinales in Basidiomycota. This fungus is a biotrophic mutualistic root endophyte isolated from the roots of xerophytic woody shrubs in the Thar desert of North Western India and was named as DSM 11827, (Verma *et al.*, 1998; Varma *et al*., 1999; Weiss *et al.*, 2004; 2016).

This fungus was named with reference to its pear-shaped chlamydospores. This was isolated from a spore of the arbuscular mycorrhizal fungus *Funneliformis* (=*Glomus*) *mosseae* from Indian desert soil (Verma *et al.*, 1998). It is an axenically cultivable root endophyte with plant growth promoting capabilities. This fungus imitates the abilities of typical arbuscular mycorrhizal fungi (AMF*). P. indica* is a broad host range root colonizing beneficial fungus and host range includes crop plants and also the model plant *Arabidopsis thaliana* (Shahollari *et al*., 2005; Deshmukh *et al*., 2006).

This root endophyte is an anamorphic strain of the Sebacinales in Basidiomycota (Weiss *et al.*, 2004; Selosse *et al*., 2007). This is a multifunctional root endophyte with biofertilizer, biocontrol and biomodulatory effects. This fungus has great value in organic farming. The use of this fungus enables the reduction of the use of water, fertilizers and pesticides in farming. (Ansari *et al.*, 2013; Rabiey *et al.*, 2017).

As reported by Ye *et al.* (2014) *P. indica* is a versatile endophytic fungus colonizing in the epidermal layers and root cortex of a plants. The mycelium of this fungus can live and propagate intracellularly and mostly in intercellular space. Johnson *et*

al. (2014), reviewed the endophytic colonization and their effects like promotion of nutrient uptake into the hosts. This in turn results in promotion in growth and the overall performance of the plants. Gill *et al.* (2016) reported an increase in the survival rate of *Chlorophytum* plantlets. This also improves the uptake of essential nutrients like phosphate, copper, iron and zinc.

As reported by several authors it interacts beneficially with a wide range of plants and is known to enhance plant growth, biomass production, phosphorus acquisition and act as a bio-protector against abiotic and biotic stress including root and leaf fungal pathogens (Shahollari *et al*., 2007; Yadav *et al.*, 2010; Wang *et al*., 2015; Varkey *et al*., 2018).

Waller *et al.* (2008) reported plants inoculated with *P. indica* showed an early expression of developmentally regulated genes. Several authors reported the earliness in flowering on *P. indica* inoculation such as in barley by Achatz *et al.* (2010), *Coleus forskohlii* (Das *et al.*, 2012), *Arabidopsis* (Kim *et al*., 2017; Pan *et al*., 2017) and black pepper (Anith *et al.*, 2018).

Anith *et al.* (2011) reported the colonization of *P. indica* in tissue cultured black pepper and its positive effect on vegetative growth. In a study conducted by Anith *et al.* (2018) they used plagiotrops as the planting material and observed colonization *by P. indica.* Persistent nature of colonization after six months of inoculation which was evidenced by the presence of chlamydospores.

P. indica root colonization in rice seedlings resulted in enhanced growth promotion with increased root and shoot biomass; and also had a greater number of secondary and tertiary roots with profuse root hairs (Ashar, 2019). An experiment conducted by Lin *et al.* (2019) demonstrated shortening of recovery period of Anthurium on inoculation with *P. indica.*

Pham *et al.* (2004) reported the growth of *P. indica* colonies was incited by *Bradyrhizobium* sp. whereas, it strongly restricted by *Pseudomonas fluorescens*. The

isolated bacteria from the association also had growth promotional and biological control effect (Glaeser *et al*., 2016).

2.4.1 *Piriformospora indica* and Disease Management

A novel approach in plant disease management is the use of beneficial fungal root endophytes as bioprotectors, bioregulators, biofertilizers and growth promoters**.** Fungal endophytes are widely used for the management of various bacterial and fungal diseases

P. indica improves defensive capacity of several crop plants against bacterial, viral nematode and fungal pathogens (Sun *et al*., 2014; Varkey, 2016; Lakshmipriya *et al*., 2017; Ashar, 2019; Chandan, 2019). Trzewik *et al*. (2020) reported that the mechanisms by which of different beneficial fungi protect the host plants against pathogens are by the competition for nutrition, antibiosis, mycoparasitism and induced systemic resistance.

P. indica colonization-controlled *Fusarium* wilt in tomato (Qiang *et al*., 2012). Nassimi *et al*. (2017) has reported that *P. indica* induced systemic resistance against rice sheath blight by decreasing the levels of hydrogen peroxide and increased activity of superoxide dismutase. Prasad *et al.* (2013) stated that *P. indica* colonization resulted in the activation of antioxidant system, which is responsibale for improvement of crop plant tolerance against biotic and abiotic stresses. *Triticum aestivum* has been found colonized with this root endophyte which resulted in providing bio protection against *B. graminis* f. sp. *tritici*, *P. herpotrichoides* and *Fusarium culmorum* (Serfling *et al*., 2007). Protection against *Fusarium verticillioides* in *Zea mays* on inoculation with *P. indica* was reported by Kumar *et al*. (2009). Plants colonized with *P. indica* were found less susceptible to *Colletotrichum falcatu* as compared to non-colonized control plants (Varma *et al*., 2012).

Hordeum vulgare plant treated with *P. indica* has found to provide protection to roots against *Fusarium culmorum* and also against shoot damage by *Blumeria graminis* (Waller *et al.,* 2005). Many root pathogens have been found to be directly inhibited by antagonistic activities of the endophytic fungus except *Fusarium culmorum* (Waller *et*

al., 2005), *P. herpotrichoides* (Serfling *et al*., 2007), *F. oxysporum* (Dolatabadi *et al.,* 2012). To the other, systemically induced resistance by *P. indica* root-colonization has also been reported for many foliar pathogens (Waller *et al.,* 2005).

According to most authors, *P. indica* more likely protects plants by inducing their systemic immunity (Serfling *et al.,* 2007; Oelmuller *et al.,* 2009; Molitor *et al.,* 2011; Pedrotti *et al.,* 2013; Johnson *et al.,* 2014; Narayan *et al.,* 2017).

Recently, in root endophyte-colonized *Hordeum vulgare* plants, a sub-set of defense-related genes was observed highly induced by leaf pathogens (Molitor *et al.,* 2011). Franken *et al*., (2012), reported that the tomato plants colonized by *P. indica*, the incidence of Verticillium wilt was reduced by 30 percent. The study conducted by Lakshmipriya *et al.* (2017) there was substantial decrease in leaf blight disease incidence in two cultivars of taro, Sree Kiran and Muktakeshi with the root endophytic fungus *P. indica.* Ashar (2019) reported that *P. indica* significantly inhibits the growth of *B. oryzae* by antibiosis, thickening and lysis of mycelia of *B. oryzae. P. indica*-primed rice seedlings and plants could significantly delay the symptoms of *B. oryzae* up to eight days of the pathogen inoculation. *P. indica*-primed rice seedlings and plants recorded the lowest lesion size and the highest disease suppression.

Hou *et al*. (2012) stated that pathogenesis-related proteins play an important role in the disease resistance response. Athira (2018) reported *P. indica* to be a biological control agent against bacterial wilt disease caused by *R. solanacearum*. An experiment conducted by Lin *et al.* (2019) showed that *P. indica* could provide protection against *Ralstonia solanacearum* by activating multiple signaling pathways involved in the defense function of *Anthurium andraeanum*. PR1 and PR5 are found up-regulated in Anthurium leaves of *P. indica* about 0.5-fold levels upon infection by *Ralstonia solanacearum*. This suggests that upon bacterial infection the endophyte plays induced systemic resistance mechanism (ISR) in plant.

Tomato plants colonized with *P. indica* repressed the amount of *Pepino mosaic virus* (Fakhro *et al*., 2010). Wang *et al*. (2015) reported that *P. indica* reduced the disease incidence and severity against *Tomato yellow leaf curl virus*. Disease incidence and severity was reduced by 26 per cent and 1.25 per cent respectively in susceptible tomato cultivar T07-1.

Reduction in the vulnerability index by increased activity of defense enzymes like peroxidase, polyphenol oxidase and by induction of pathogenesis related proteins against *Black eye cowpea mosaic virus* in cowpea was noted on colonisation by *P. indica* (Alex, 2017; Chandran, 2019). Pre-inoculation of *P. indica* inhibited *Cymbidium mosaic virus* and *Odontoglossum ring spot virus* in orchid, *Dendrobium* (Safeer, 2019).

2.5 COMBINED INOCULATION OF *Piriformospora indica* WITH OTHER ORGANISMS

Mixed inoculation or a consortium of bioagents always would have added advantage over the individual application and for increasing the spectrum of action and efficiency of bio-inoculants, they can be used as mixed inoculum or consortium with more than one bio-agents as a formulation (Pierson and Weller, 1994; Vidhyasekarn and Muthamilan, 1995; Schisler *et al*., 1997; Janisiewicz, 1988; Jetiyanon and Kloepper, 2002; Slininger *et al*., 2010). Klopper *et al.* (2014) suggested that mixing strains of biological disease control agents can increase the repeatability of efficacy.

Meena *et al.* (2010) reported that the single inoculation of *P. indica* and phosphate solubilizing *Pseudomonas striata* had a lesser effect on plant growth and yield of chickpea than their combined application. An increment of 8.8 fold in dry root weight and 8.6 in dry shoot weight of tomato plants and control of Fusarium wilt using inorganic carrier- talc based formulations of two fluorescent pseudomonad strains (R62 and R81) and *P. indica* in controlled glass house experiment was reported. A similar observation was noticed in the field also (Sarma *et al*., 2011).

Increased growth of mung bean under field conditions was reported when applied with a consortium containing *P. indica* and two *Pseudomonad* strains (R62 and R81). Increase in dry root and shoot weight, number of nodules produced and number of pods harvested from mung bean plants were noted in the consortial treatment compared to the control (Kumar *et al.,* 2012). Saxena *et al*. (2015) reported synergistic effects of co-inoculation of phosphate solubilizing bacteria and the endophytic fungus has been demonstrated in pot culture experiments involving chick pea

An experiment conducted by Anith *et al.* (2015) reported that tomato seedlings when inoculated with a mixture of *P. indica* and *B. pumilus*, significantly increased growth of them as compared to the individual application of the two biological agents.

Enhanced artemisin content in the medical plant *Artemisia annua* on on application of dual biological consortium containing *P. indica* and *Azotobacter chroococcum* was reported by Arora *et al.* (2016). Increased contents of chlorophyll and nutrients such as nitrogen and phosphorous was noticed which resulted in the increased overall productivity. Enhancement of metabolite content in *Artemisia annua* occurred on dual inoculation with the bacterial and fungal bioagents under *in vitro* conditions also (Arora *et al*., 2018).

Antagonistic reaction against *P. indica* by *Trichoderma harzianum* was proved and they could be used as dual inoculants in tissue cultured black pepper, if the root endophyte, *P. indica* was applied at an early hardening stage, followed by the application of *Trichoderma harzianum* during transplanting to the field (Anith *et al.,* 2011). A study conducted by Athira (2018) using combined application of *P. indica* with rhizobacteria and endophytic bacteria showed combined inoculation resulted in reduced suppression of bacterial wilt incidence in two varieties of tomato, Naveen and Vellayani Vijay except in a few cases. Nandana (2019) reported application of *P. indica* and *P. fluorescens* PN026 in combination resulted in the early flowering, highest root fresh weight and dry weight, higher number of leaves and branches.

Materials and Methods

3. MATERIALS AND METHODS

The experiment on the "Plant growth promotion and foot rot disease suppression in black pepper using fungal and bacterial endophytes" was carried out during the period from 2018-2020 in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram. The details of the materials used and methods followed in the present study are presented in this chapter.

3.1 MICROORGANISMS AND CULTURE CONDITIONS

Endophytic bacterial isolates *Bacillus velenzensis* PCSE 10 and *Rhizobium radiobacter* PCRE 10, isolated from *Piper colubrinum* in a previous study at the Department of Agricultural Microbiology were used in this study (Kollakkodan, 2017). Bacterial isolates were routinely cultivated on Nutrient Agar (NA) medium and maintained on NA slants under refrigeration (Plate 1). The endophytic fungus *Piriformospora indica*, gifted by Dr. Ajit Varma, former Professor, Jawaharlal Nehru University, New Delhi, India, was routinely cultivated on Potato Dextrose Agar (PDA; pH 7) medium at 28°C and was maintained on PDA slants under refrigeration.

3.2 PATHOGEN

3.2.1 Isolation

Isolation of pathogen was carried out from *Phytophthora capsici* infected black pepper leaves collected from pepper gardens of Instructional Farm, College of Agriculture, Vellayani. Infected portion on leaves were cut into small pieces and washed thoroughly in sterile distilled water for 5-6 times. The smaller bits of infected leaves were then kept along the edges of the half -filled plates of Potato Dextrose Agar (PDA). Cephalexin was supplemented in PDA media in order to prevent bacterial contamination. Plates were kept for incubation in inverted position for 2-3 days at 28^{o}C for mycelial development around the bits placed on media. Smaller bits of mycelial growth were cut out and placed on

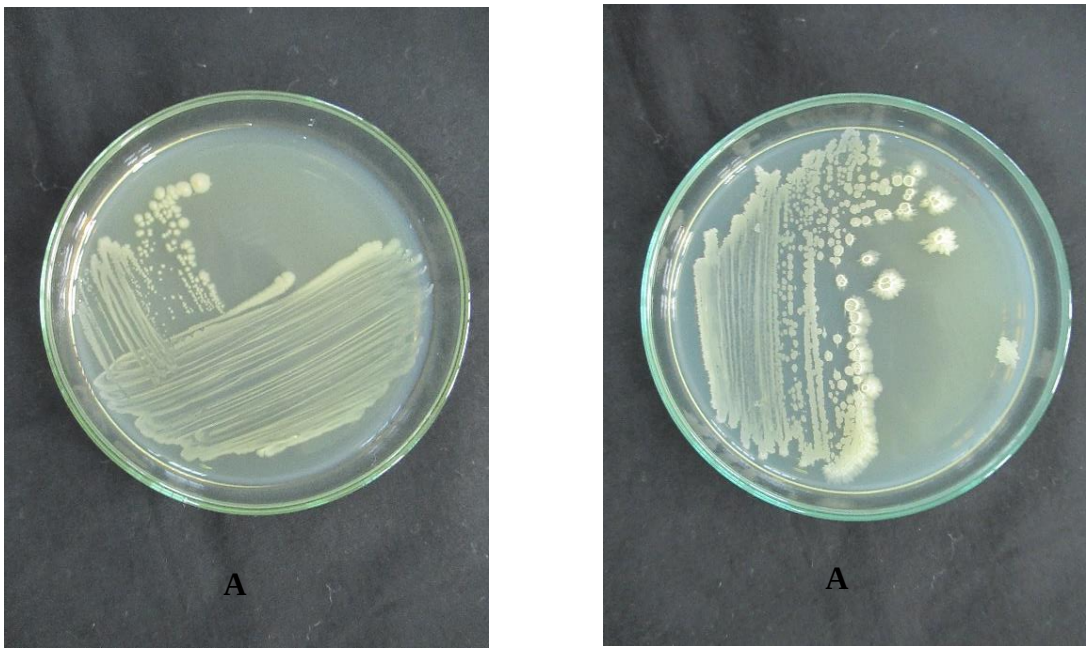


Plate 1. Bacterial endophytes used in this study

A. **A) *Rhizobium Radiobacter* PCRE 10**

B*) Bacillus velezensis* PCSE 10

PDA plates to get pure culture of the pathogen. Mycelium was aseptically transferred to sterile PDA slants and then maintained in a desiccator chamber for further studies.

3.2.2 Pathogenicity

Healthy leaves of black pepper were collected from Instructional farm, College of Agriculture, Vellayani and washed well in water. They were allowed to dry in a laminar air flow chamber. Detached healthy leaves were inoculated with isolated pathogen by placing mycelial discs on the lower leaf surface after providing a pinprick. Moist sterile cotton was placed over mycelial disc to maintain humidity. The proximal end of leaves was covered with moist cotton to avoid drying of leaves. Development of leaf lesion was observed 1-2 days after pathogen inoculation.

3.3 *IN VITRO* STUDIES

3.3.1 *In vitro* antagonism between *Piriformospora indica* and bacterial strains

Dual culture plate assay was carried out as described by Anith *et al.* (2015) to study whether the endophytic bacteria showed any inhibition towards the growth of *P. indica.* A mycelial disc (4 mm dia) was cut out from a fresh culture of *P. indica* grown on PDA and placed at the centre of Petri plate containing agar medium. Inoculated plates were incubated at 28^0C for five days. Single colonies of *Bacillus velezensis* PCRE 10 and *Rhizobium radiobacter* PCRE 10 were obtained by streak plating on Nutrient agar medium. After five days of incubation of *P. indica* plates, using an inoculation loop a heavy inoculum from single colony of the bacterial strain was applied as a band of 1.5 cm length at two opposite edges of the Petri plates equidistantly. Plates were further incubated at 28^0C and presence of the inhibition zone was noticed.

3.3.2 *In vitro* antagonism of bio agents against *Phytophthora capsici*.

Dual culture plate assay was carried out in PDA plates to assess the direct antagonistic effect of bio agents against the foot rot pathogen. Dual culture assay of

endophytic bacteria against *P. capsici* was carried out following the procedure given by Kollakkodan *et al*. (2017). A mycelial disc (4 mm dia) was cut out from a fresh culture of *P. capsici* grown on PDA and placed at the centre of Petri plate containing agar medium. Inoculated plates were sealed with cling film and incubated at 28^0C for five days. Single colonies of *Bacillus velezensis* PCRE 10 and *Rhizobium radiobacter* PCRE 10 were obtained by streak plating on Nutrient agar medium. After five days of incubation of *P. capsici* plates, using an inoculation loop a heavy inoculum from single colony of the bacterial strain was applied as a band of 1.5 cm length at two opposite edges of the Petriplates equidistantly. Plates were further incubated at 28^0C and presence of the inhibition zone was noticed. Dual culture assay was also conducted to assess the antagonistic activity of *P. indica* against *P. capsici.* One mycelial disc (4 mm dia) each from both the pathogen and endophytic fungus obtained from freshly grown PDA plates was placed at two opposite sides on PDA medium one cm away from the edge. Plates were incubated at 28^0 C for 5 to 6 days. Presence of the inhibition zone was noticed.

Growth inhibition by *P. indica* on *P. capsici* was quantified using the following formula from five independent dual culture plates.

$$\text{Growth inhibition} = \frac{C-T}{T} \times 100$$

Where C is the diameter (cm) of *P. capsici* growth on control plates, T is diameter of *P. capsici* growth on dual culture plates.

Hyphal interaction was studied according to Anith *et al*. (2011). *P. capsici* and *P. indica* were grown on Potato Dextrose Agar (PDA) medium for four and seven days respectively. A sterile cellophane membrane of 10 cm diameter was pressed gently on to the surface of PDA plate. A mycelial disc of four mm diameter was cut from the culture plate of *P. capsici* and inoculated on to the plate over the cellophane membrane along a diameter of the plate at a distance of 2.5 cm from the centre. A similar mycelial disc from *P. indica* culture was cut out and placed opposite the growing *P. indica* culture in the test

plate along the diameter at an equal distance from the centre. The plate was incubated for five more days at 28°C and interacting zones of the fungal cultures were cut out by lifting the cellophane membrane. The interacting zone after staining with lactophenol cotton blue was observed under light microscope.

3.3.3 Screening of disease suppression by detached leaf assay

Detached leaf assay was carried out to evaluate the ability of antagonistic bacterial isolates to suppress the infection of *P. capsici*. The bacterial inoculum used in the study was prepared by growing them in agar plates as described by Varkey *et al*. (2018). Using a sterile loop, bacterial cells were collected from single colony and heavily cross streaked on nutrient agar plates. The plates were incubated overnight at 28 ± 2 °C. The plates were then drenched with ten ml of sterile distilled water. Using a sterile glass spreader the bacterial cells were scrapped out. The cell suspension was collected aseptically into a sterile glass vial. Mature leaves from the black pepper variety Panniyur 1 were collected from Instructional farm, College of Agriculture, Vellayani and washed well in water. They were allowed to dry in a laminar air flow chamber and dipped in the bacterial suspension separately. Leaves sprayed with sterile water served as control. The leaves are allowed to get dry in laminar air flow chamber. A mycelial disc (8 mm) from freshly grown *P. capsici* was taken and placed in the lower side of leaf surface after providing a pinprick. Moist sterile cotton was placed over mycelial disc to maintain humidity. The petiole end of leaves was also covered with moist cotton to avoid drying of leaves. Pathogen inoculated leaves were also kept as control. Lesion size in leaves and time taken for appearance of lesion was taken to assess the effectiveness of bacterial endophytes. The experiment was designed as CRD with five replications.

Relative inhibition (RI) was calculated on the basis of growth in control leaves as:

$$RI\ (\%) = \frac{\text{Radial growth in control - Radial growth in samples x 100}}{\text{Radial growth in control}}$$

3.3.4 Characterization of the bio agents for the production of plant growth promoting and disease suppressing metabolites.

The bacterial bio agents were tested for the production of metabolites like IAA (Gordon and Weber, 1951) and ammonia (Cappuccino and Sherman, 1992).

3.3.4.1 IAA production

Indole Acetic Acid was estimated as per the procedure described by Gordon and Weber (1951) employing Salkowski method. One set each of 100 ml nutrient broth was inoculated with endophytic bacteria with or without tryptophan (0.1%) and incubated at 28°C for 48 hours with constant shaking in a rotary shaker (Scigenics, India) at 100 rpm for obtaining log phase. Uninoculated broth was taken as control. After five days of incubation the broth cultures were transferred to centrifuge tubes and centrifuge at 4500 rpm for 20 min at 4°C. Then one ml of the supernatant was transferred to a test tube and two ml Salkowski reagent was added to it. Salkowski reagent was prepared by mixing two ml 0.5M $FeCl_3$ and 49 ml water and 49 ml 70% perchloric acid. The tubes were kept in dark for 25 min for colour development. Absorbance was measured at 530nm.The absorbance of the samples obtained was plotted against a standard to determine the concentration of IAA produced.

Standard curve of IAA was made with concentrations of 0, 5, 10, 20, 50, and 100 µg/ml (ppm). Stock solution of 1000 µg/ml concentration was made by adding 10 mg IAA to a glass beaker containing 10 ml acetone. A series of amber vials were labeled with the dilution series. Test tubes were labeled with each standard and two ml Salkowski Reagent was transferred including control of pure media. The tubes were kept in dark for 25 mins for colour development. Absorbance was measured at 530nm.

3.3.4.2 Ammonia production

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water separately and incubated

for 48 - 72 h at 36 ± 2°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman, 1992).

3.4 *IN VIVO* STUDIES

3.4.1 Plant growth promotion in bush pepper (pot culture experiment)

An experiment was conducted to evaluate the efficacy of endophytic bacteria, *P. indica* and their combination in growth promotion of bush pepper. Bush pepper plants were raised at Coconut Research Station, Balaramapuram in a naturally ventilated greenhouse condition (Plate 2). Later on, plants were transferred to earthen pots.

3.4.1.1 Bacterial inoculum preparation

The bacterial inoculum used in the study was prepared by growing them in agar plates as described by Varkey *et al*. (2018). Using a sterile loop, bacterial cells were collected from single colony and heavily cross streaked on nutrient agar plates. Then the plates were incubated overnight at 28 ± 2 °C. The plates were drenched with ten ml of sterile distilled water. Using a sterile glass spreader the bacterial cells were scrapped out. The cell suspension was collected aseptically into a sterile glass vial. The OD of cell suspension was adjusted to 1.0 at 660 nm using sterile distilled water resulting in uniform suspension of approximately 10^7cfu/ ml.

3.4.1.2 Cultivation and application of P. indica

Mass multiplication of root endophytic fungus *P. indica* was carried out by the procedure given by Anith *et al*. (2018). The fungal inoculum development was carried out in 500ml capacity Erlenmeyer flasks containing 100ml of PDB (pH 7). PDB was inoculated with three mycelial discs (8mm) from a freshly grown PDA plates and then incubated at 28°C for 10 days with constant shaking in a rotary shaker (Scigenics, India) at 105 rpm (Plate 3). The mycelium was harvested by filtration after 10 days using a sterile muslin cloth

and then washed twice with sterile water to remove media contents. The weight of harvested mycelium was determined. Freshly harvested mycelium was mixed with sterile vermiculite to get a final concentration of one percent (w/v) fungal mycelial mass (Anith *et al.,* 2015). Vermiculite used for mixing was previously sterilized by autoclaving for one hour at 121°C for three consecutive days. For inoculation with the fungus 50g of the inoculum prepared in sterile vermiculite was added in the planting holes. The rooted cuttings were laid over the fungal inoculum so that close contact with root was ensured.

3.4.1.3 Production of bush pepper

Young healthy lateral branches (one year old) from high yielding vines were collected and care was taken not to dry them out. The collected laterals were later pruned to 2-3 nodes. Flag leaf was retained and all other leaves were removed. A sharp slanting cut was made at the basal portion and dipped in bacterial cell suspension for 20 minutes with intermittent shaking in the case of treatments involving endophytic bacteria. Fungal inoculum was applied in the planting holes.

Polythene bags were filled with equal proportion of sand, unsterile soil and farm yard manure. Cuttings were kept in a shade house for three months for establishment. Watering was done regularly. After three months the rooted cuttings were transferred to larger earthen pots (25cm dia., 30cm height) filled with a mixture of soil, sand and farm yard manure in equal proportions. The polythene bags were removed carefully without disturbing the root system. Fungal mycelial mass mixed with sterile vermiculite (1% w/v) was applied at the small cavity made for keeping plants. Fifty ml bacterial cell suspension (10^7cfu/ ml) was poured into the root zone in the case of treatments requiring bacterial treatment. Pots were arranged in completely randomized design (CRD). Each treatment was having five replications. Water soluble chemical fertilizer (N:P:K- 19:19:19) solution (0.5%)was given 50 ml to each pot at 15 days interval starting from 30th day of planting (Anith *et al*., 2018).

3.4.1.5 Details of Pot Culture Experiment

Location	: College of Agriculture, Vellayani.
Crop	: Bush pepper
Variety	: Panniyur-1
Design	: Completely Randomized Design
Treatments	: 6
Replications	: 5
Number of plants/replication	: 1

3.4.1.6 Treatments

T_1: *Piriformospora indica*

T_2: *Bacillus velezensis* PCSE 10

T_3: *Rhizobium radiobacter* PCRE 10

T_4: Control

T_5: Combination of *Piriformospora indica* and *Bacillus velezensis* PCSE 10

T_6: Combination of *Piriformospora indica* and *Rhizobium radiobacter* PCRE 10

3.4.1.7 Observations

Biometric parameters like number of leaves and leaf length were taken at monthly intervals after transplanting. Spikes were harvested at maturity. Observations like number of spikes harvested per plant and mean spike length (cm) were also taken.

3.4.1.7.1 Percentage establishment of bush pepper

The percentage establishment was calculated after three months of planting in polythene bag using the following formula.

$$\% \text{ establishment of bush pepper} = \frac{\text{Number of rooted cuttings}}{\text{Total number of cuttings}} \times 100$$

3.4.1.7.2 Number of leaves per plant

Total number of leaves per plant was taken at monthly intervals from the day of transplanting of pepper plants to larger pots.

3.4.1.7.3 Leaf area (cm^2) per plant

Allometric model proposed by Kandiannan *et al*. (2002) was utilized for estimating leaf area. Leaf length was taken at monthly interval from the day of transplanting to larger pots. The length from the point at which the lamina is attached to the petiole to the tip of the leaf was taken as leaf length which was measured in centimeter (cm). Leaf area of black pepper variety Panniyur-1 was calculated by applying the specific mathematical formula $LA = 0.7114\ (LL)^{1.8409}$, where LA is the leaf area (cm^2) and LL is the leaf length in cm.

3.4.1.7.4 Yield characteristics

Spikes were harvested at maturity. Observations like number of spikes harvested per plant and mean spike length (cm) were also recorded.

3.4.1.7.5 Detection of P. indica colonization

After one month and six months of transplanting, five root samples each from the treated and untreated plants were excavated from the pots without damaging the plants. Staining was done as described by Anith *et al*. (2011). The collected roots were washed thoroughly in running water to remove soil and other debris. Then the roots were cut into bits of 1 cm length. The roots were then kept in freshly prepared 10 % KOH solution for 15 mins and then boiled for 5 min so that the roots become softened and tannins and phenols were washed out from roots. After that the roots were washed in distilled water twice. Acidification of root bits was carried out with 1 M HCl for 5 min and stained in

0.2% lactophenol-trypan blue for 15min. Lactophenol solution was utilized for de-staining. Roots bits after de-staining can be taken for the observation under compound bright field microscope. Root colonization can be observed as distinct pear shaped chlamydospores inside the root cortex. Percentage of root colonization was estimated using following formula.

$$\% \text{ root colonization} = \frac{\text{Number of root segments colonized}}{\text{Total number of root segments observed}} \times 100$$

3.4.2 Disease suppression in the established bush pepper plants

An experiment was conducted to evaluate the efficacy of endophytic bacteria, *P. indica* and their combination in controlling the foliar infection caused by *P. capsici* in black pepper (Plate 2).

3.4.1.5 Details of Pot Culture Experiment

Location	: College of Agriculture, Vellayani.
Crop	: Bush pepper
Variety	: Panniyur-1
Design	: Completely Randomized Design
Treatments	: 8
Replications	: 5
Number of plants/replication	: 1

3.4.1.6 Treatments

T_1: *Piriformospora indica*

Plate 2. General view of black pepper nursery

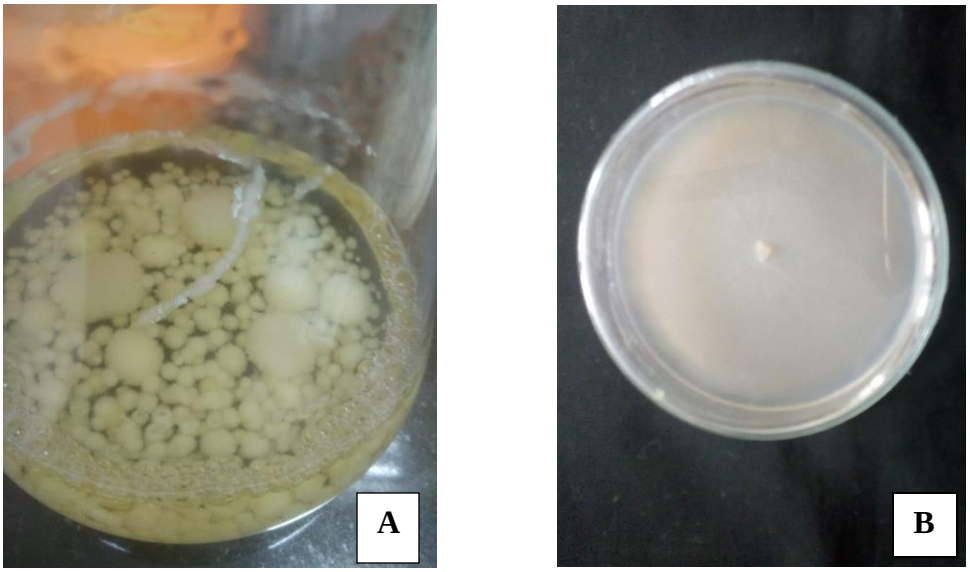


Plate 3- Root endophyte, *Piriformospora indica*

A- Mass multiplication in Potato dextrose broth at pH 7.0

B- Mycelial growth on Potato dextrose agar

T_2: *Bacillus velezensis* PCSE 10

T_3: *Rhizobium radiobacter* PCRE 10

T_4: Combination of *Piriformospora indica* and *Bacillus velezensis* PCSE 10

T_5: Combination of *Piriformospora indica* and *Rhizobium radiobacter* PCRE10

T_6: Pathogen inoculated control

T_7: Chemical control (spraying with 0.2% copper oxychloride)

T_8: Absolute control

3.4.2.1 Pathogen Inoculation

Virulent strain of *Phytophthora capsici* was used for inoculation on to healthy intact leaves of treated bush pepper plants kept in earthen pots. Mycelial plugs from fully grown PDA plates were made using 4 mm cork borer. Inoculation of *P. capsici* was done on lower leaf surface of intact leaves of eight months old bush pepper plants. The mycelial plugs were covered with thin layer of moist cotton and leaves were covered with polythene covers to provide humidity. Inoculation was done in all plants except the absolute control. Development of lesion was observed and the lesion size on inoculated leaves was recorded.

3.4.2.1 Application of COC

COC (0.2%) was sprayed onto the foliage one week before pathogen inoculation.

3.4.2.1 Observations

3.4.2.1.1 Lesion Size on Inoculated Leaves

Lesion size on inoculated leaves was measured by measuring the length of lesion developed in centimeters at 24 hours interval up to six days after inoculation.

3.4.2.1.2 Disease Index

Disease index was calculated based on a scoring from 0-5 based on the lesion size on inoculated leaves for scoring *P. capsici* induced foliar infection in bush pepper. The disease index was calculated based on the lesion size values taken at 6 days after inoculation. Scale for scoring *Phytophthora capsici* induced foliar infection in black pepper (Plate 4) is shown below.

Score	Lesion size (cm)
0	No Lesion
1	0.1 – 2 cm
2	2.1 – 3 cm
3	3.1 – 4 cm
4	4.1 – 5 cm
5	> 5 cm

Disease index (DI) was calculated using the formula:

$$DI = \frac{\text{Sum of individual ratings}}{\text{Number of leaves assessed x Maximum grade used}} \times 100$$

3.5 STATISTICAL ANALYSIS

Statistical analysis was done using the package OPSTAT available with the online portal of CCS, HAU, Hisar. Data obtained in the experiments were analysed using ANOVA and one factorial completely randomised design (CRD). Least significant differences were calculated at the 5% probability level of significance for comparison of different treatments.

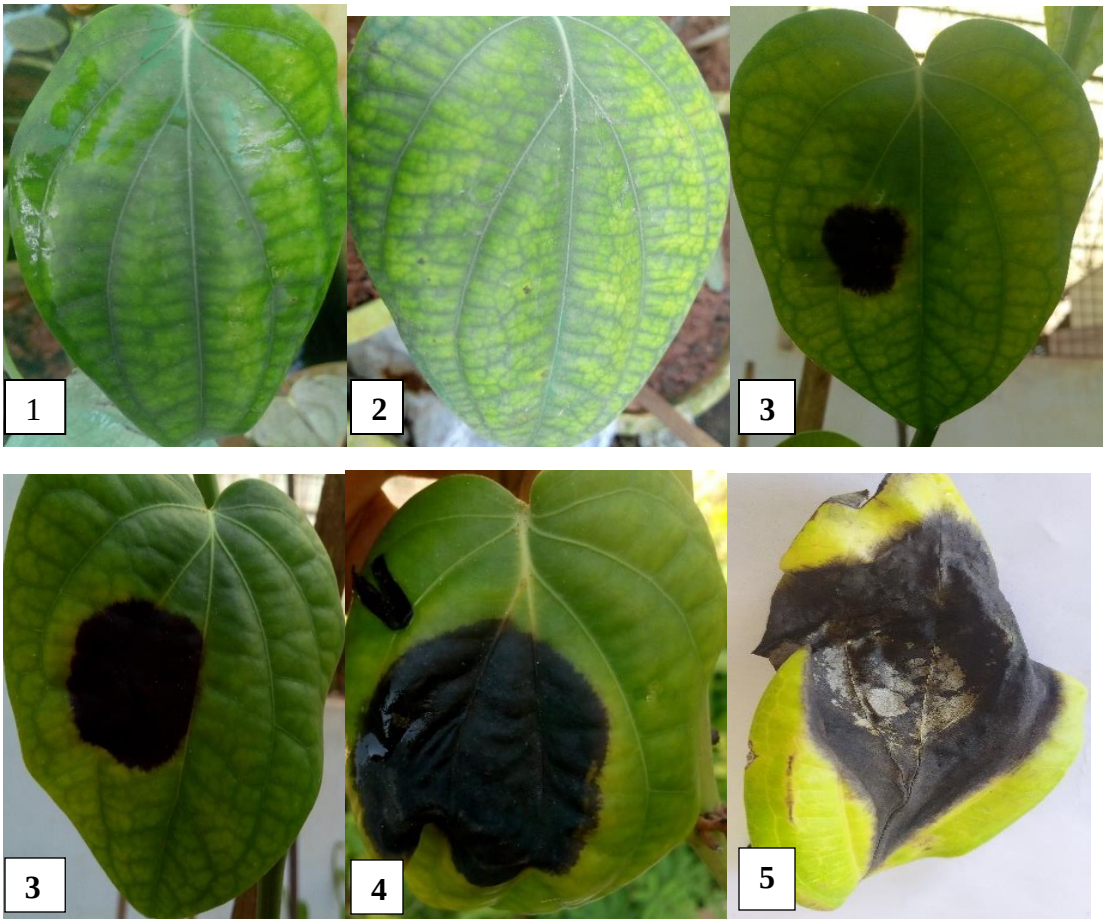


Plate 4 - Scale for scoring leaf lesion developed by *Phytophthora capsici* on artificial inoculation

Results

4. RESULTS

The present study on "Plant growth promotion and foot rot disease suppression in black pepper using fungal and bacterial endophytes" was conducted during the period from 2018-2020 in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. The results based on statistically analyzed data pertaining to the experiment conducted during the course of investigation are presented below.

4.1. ISOLATION OF PATHOGEN

Virulent strain of *Phytophthora capsici* was isolated from infected leaves. This when inoculated onto healthy leaves of black pepper caused characteristic black coloured lesions (Plate 5).

4.2. *IN VITRO* STUDIES

4.2.1. ***In vitro*** **ANTAGONISM BETWEEN** ***Piriformospora indica*** **AND BACTERIAL STRAINS**

Dual culture plate assay was done on PDA plates as both the fungal endophyte and the bacterial agents could grow well on PDA media. The zone of inhibition was measured in 5^{th} day after the bacterial inoculation. The results showed that *P. indica* growth was not inhibited by *Rhizobium radiobacter* PCRE10. However, *Bacillus velezensis* PCSE10 produced an inhibition zone of 4 mm (Table 1, Plate 6).

4.2.2. ***In vitro*** **ANTAGONISM OF BIO AGENTS AGAINST** ***Phytophthora capsici.***

The observations on antagonistic effect of two bacterial endophytes and *Piriformospora indica* tested against *Phytophthora capsici* are given in table 2. The results of the dual culture plate assay showed that *B. velezensis* PCSE10 exhibited more antifungal activity against the foot rot pathogen evidenced by presence of inhibition zone (Plate 7). The zone of inhibition was measured in 6^{th} day after the bacterial inoculation.

Table 1. Mycelial growth inhibition of *P. indica* by bacterial strains in dual culture plate assay

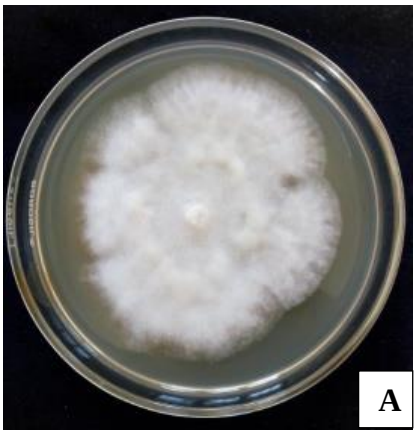
Bacterial strain	ZONE OF INHIBITION (mm) *
Bacillus velezensis PCSE 10	4.00
Rhizobium radiobacter PCRE 10	Nil

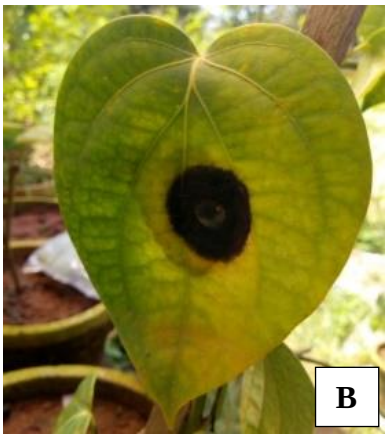
Mean of eight observations from four dual culture plates. Each plate represents single replication (n=4)
*PDA = Potato dextrose agar

Table 2. Mycelial growth inhibition of *P. capsici* by bacterial strains in dual culture plate assay

Bacterial strain	ZONE OF INHIBITION (mm) *
Bacillus velezensis PCSE 10	4.67±0.333
Rhizobium radiobacter PCRE 10	1.03±0.226
SE(m)	0.28
C.D.	**0.90**

Mean of eight observations from four dual culture plates. Each plate represents single replication (n=4) *PDA: Potato dextrose agar; DAI: Days after inoculation





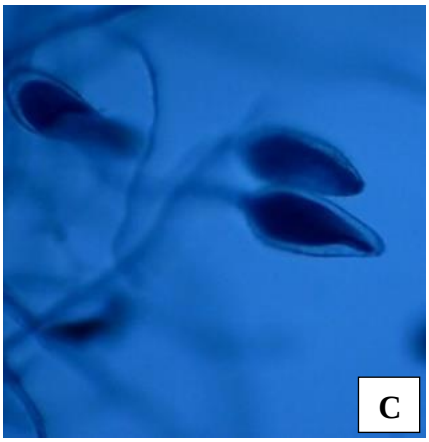
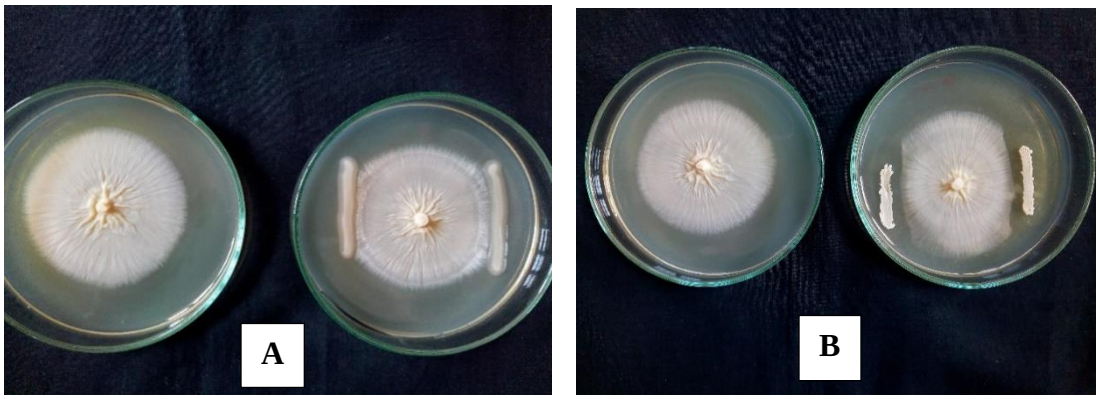


Plate 5. The pathogen: *Phytophthora capsici*

A: Growth on Potato Dextrose Agar

B: Symptom developed on black pepper leaf on artificial inoculation

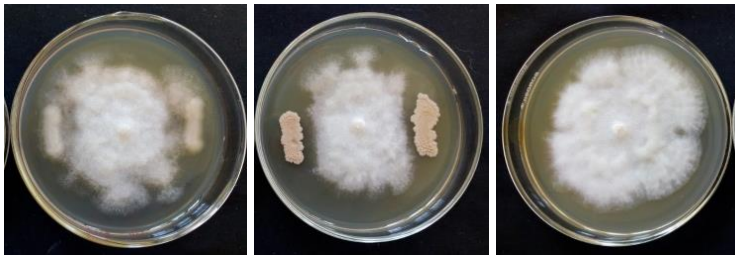
C: Papillate, caducous sporangium with long pedicel



Plates 6. ***In vitro*** **assessment of compatibility between bacterial bioagents and** ***Piriformospora indica*** **by dual culture plate assay**

A) ***Rhizobium Radiobacter*** **PCRE 10**

B) ***Bacillus velezensis*** **PCSE 10**



PCRE 10 **PCSE 10** **CONTROL**

Plate 7. *In vitro* inhibition of *Phytophthora capsici* by endophytic bacterial isolates on dual culture plate assay on potato dextrose agar (PDA) medium

The results showed that *Phytophthora capsici* growth was inhibited by *Rhizobium radiobacter* PCRE10 lesser as compared to that of *Bacillus velezensis* PCSE10 (Table 2).

Dual culturing of the *P. indica* and *P. capsici* showed that *P. indica* grew over the mycelium of *P. capsici* and inhibition of the mycelial growth in *P. indica* was 25% after 17 days of growth in PDA (Table 3). Microscopic examination of the interacting zone of the fungi revealed that both fungi grew well in the interaction zone. Chlamydospores of *P. indica* was distributed in the interaction zone. Intact hyphae of *P. capsici* was also present (Plate 8).

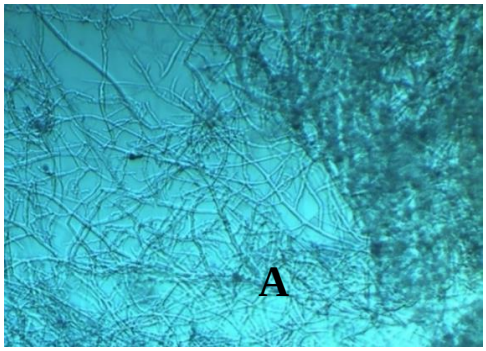
4.1.3. DETACHED LEAF ASSAY

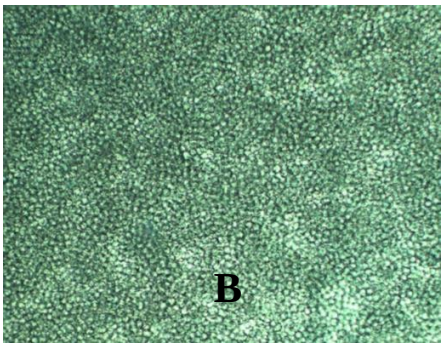
The lesion size (cm) on inoculated leaves of black pepper var Panniyur -1 sprayed with the individual isolates was measured four days after pathogen inoculation (Table 4). There was significant difference in lesion size on control leaves sprayed with sterile water and leaves sprayed with the selected isolates (Plate 9). The minimum lesion size was observed in leaves treated with *Rhizobium radiobacter* PCRE10 with a lesion size of 1.43 cm which caused 52.33 % disease suppression over control. Leaves treated with is *Bacillus velezensis* PCSE 10 produced lesion size of 2.18 cm and caused 27.33% disease suppression over control. The maximum lesion size was observed in control leaves sprayed with distilled water (3.0 cm). In control leaves and leaves treated with isolates the lesion development was observed within 38 hours of pathogen inoculation.

4.1.4. CHARACTERIZATION OF THE BIO AGENTS FOR THE PRODUCTION OF PLANT GROWTH PROMOTING AND DISEASE SUPPRESSING METABOLITES.

4.1.4.1 Estimation of Indole Acetic Acid Production by the bio agents

IAA produced by the bacterial bio agents was estimated five days after inoculation. All the isolates produced significant quantity of IAA under *in vitro*





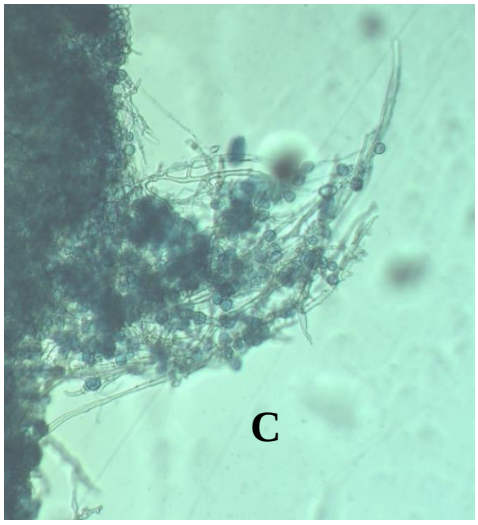
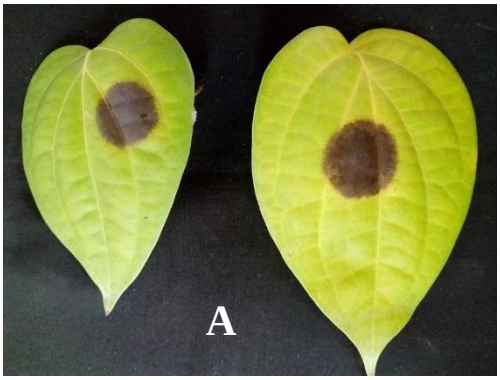


Plate 8- Dual culture on cellophane membrane

A- *Phytophthora capsici;* B- *Piriformospora indica*; C- Interaction zone between *P. capsici* and *P. indica*



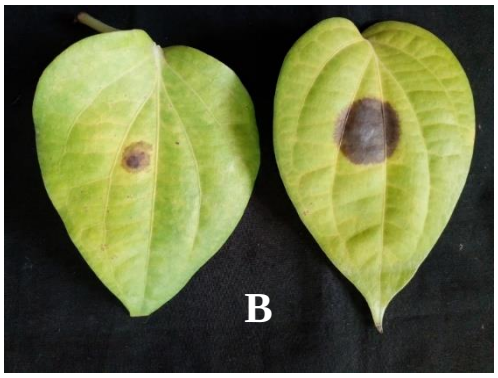


Plate 9 Detached leaf assay

A- Leaves treated with *Bacillus velezensis* PCSE 10 VS control

B- Leaves treated with *Rhizobium radiobacter* PCRE 10 VS control

Table 3. Mycelial growth inhibition of *P. capsici* by *P. indica*

Treatment	Colony diameter of *P. capsici* (cm)*		
	4 DAI	8 DAI	17 DAI
Piriformospora indica dual plate	1.32	2.50	3.52
Control	1.30	2.72	4.70
% mycelial growth inhibition	-1.92	8.25	25.00

*Mean of four independent observations

Table 4. Leison size on detached leaves of black pepper variety Panniyur – 1 challenge inoculated with *P. capsici*

Bacterial strain	LEISON SIZE (cm) *
Bacillus velezensis PCSE 10	2.18± 0.16 [b]
Rhizobium radiobacter PCRE 10	1.43± 0.24 [c]
Control	3.00±0.15 [a]
SE(m)(±)	0.19
CD (0.05)	**0.58**

*Mean (± SD) of six replications taken four days after challenge inoculation

conditions**.** *Rhizobium radiobacter* PCRE 10 was found to produce 4.616 µg/ml and 8.793 µg/ ml IAA without and with L- Trypthophan respectively. *Bacillus velezensis* PCSE 10 was found to produce 3.768 µg/ml and 4.920 µg/ml IAA in absence and with L-Tryptophan respectively (Table 5).

4.1.4.2 Ammonia production by bio agents

In vitro assessment of ammonia production by *Rhizobium radiobacter* PCRE 10 and *Bacillus velezensis* PCSE 10 was done (Plate 10, Table 6). Among the plant growth promoting traits screened, it was observed that isolate *Rhizobium radiobacter* PCRE 10 was positive for Ammonia as noted by the development of brown to yellow colour.

4.2. *In vivo* STUDIES

4.2.1. PLANT GROWTH PROMOTION IN BUSH PEPPER (POT CULTURE EXPERIMENT)

4.2.1.1. Percentage establishment of bush pepper (%)

There was no significant difference in percentage of establishment of bush pepper among the treatments (Table 7).

4.2.1.2. Number of leaves per plant

Highest number of leaves was recorded in the plants treated either with single or combination of *P. indica* and *Rhizobium radiobacter* PCRE 10(Table, 8). In the observation taken at first month after transplanting higher number of leaves (14) was recorded in the treatment with combined application of *P. indica* and *Rhizobium radiobacter* PCRE 10 and the values were statistically on par with those treated with single application of *Rhizobium radiobacter* PCRE 10 (12.66). On second month after transplanting, highest number of leaves (16.33) was observed in the treatment with combined application of *P. indica* and *Rhizobium radiobacter* PCRE 10 and the values

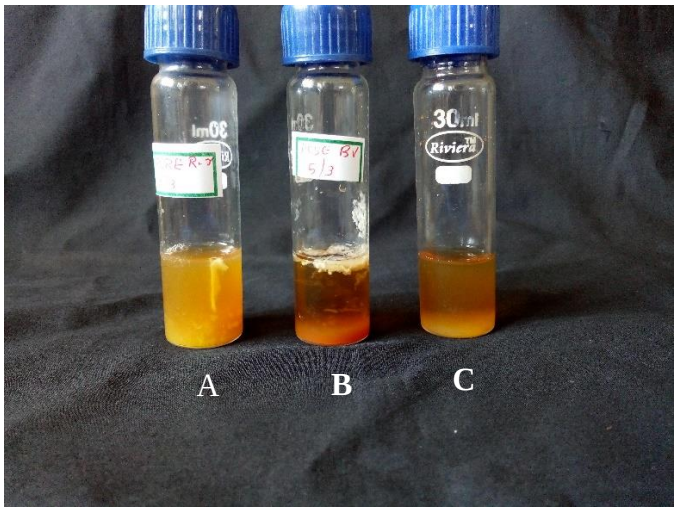


Plate 10. Ammonia production by bacterial endophytes

A*) Rhizobium radiobacter* PCRE 10; B) *Bacillus velezensis* PCSE 10; C) Control

Table 5. IAA production by bacterial endophytes

Bacterial strain	IAA (µg mL^{-1}) *	
	Without L Tryptophan	**With L Tryptophan**
Bacillus velezensis PCSE 10	3.76	4.92
Rhizobium radiobacter PCRE 10	4.61	8.79
C.D.	0.52	0.84
SE(m)	0.17	0.27

*Mean of six replications

Table 6. Ammonia production by bacterial endophytes

Bacteria	Ammonia production
Bacillus velezensis PCSE 10	_
Rhizobium radiobacter PCRE 10	+

+ Present – Absent

Table 7. Percentage of establishment of bush pepper on treatments with various bioagents

Treatments	Percentage of establishment of bush pepper (%)
Piriformospora indica	46.20 ± 8.08
Bacillus velezensis PCSE 10	39.20 ± 6.60
Rhizobium radiobacter PCRE 10	46.20 ± 8.08
Control	46.20 ± 8.08
P. indica and *B. velezensis* PCSE 10	33.00± 0.00
P. indica and *R. radiobacter* PCRE 10	33.00 ± 0.00
SEm (±)	**6.32**
CD (0.05)	**NS**

*Mean (± SD) of five replications having three plants per each replication

were statistically on par with those treated with single application of *Rhizobium radiobacter* PCRE 10 (14.33) (Table 8). Observations taken in third, fourth and fifth months after transplanting, higher number of leaves were recorded in the treatment with combined application of *P. indica* and *Rhizobium radiobacter* PCRE 10, followed by single application of *P. indica.* Observations taken in sixth and seventh month after transplanting, higher number of leaves was recorded in plants treated with *Rhizobium radiobacter* PCRE 10 alone (56 and 84 leaves respectively) and the values were statistically on par with those treated with combined application of *P. indica* and *Rhizobium radiobacter* PCRE 10.

4.2.1.3. Leaf area per plant

A significantly increasing trend was observed in leaf area per plant of the plants treated with treated with combined application of *P. indica* and *Rhizobium radiobacter* PCRE 10 (Table 9). Maximum leaf area per plant was recorded in the treatment with combination of *Piriformospora indica* and *Rhizobium radiobacter* PCRE 10 from fourth month after transplanting and it was statistically on par with the treatment consisting of *Piriformospora indica* alone.

4.2.1.4. Number of spikes harvested per plant

Observation on number of spikes harvested was taken eight months after transplanting. When the number of spikes harvested was analyzed statistically, there were no significant difference observed among the treatments. The highest value was recorded in the treatment with combination of *Piriformospora indica* and *Rhizobium radiobacter* PCRE 10 (Table 10).

4.2.1.5. Mean spike length

Mean spike length of harvested berries was analyzed statistically, there was significant difference observed among the treatments. Application of *P. indica* alone resulted in the highest mean spike length (Table 11).

Table 8. Number of leaves of bush pepper plants treated with various bioagents

Treatments	No. of leaves/plant*							
	Time of observation							
	1 MAT	2 MAT	3 MAT	4 MAT	5 MAT	6 MAT	7 MAT	8 MAT
Piriformospora indica	10. 33	11.67	16.33	21.00	26.00	35.00	48.33	54.00
Bacillus velezensis PCSE 10	7.00	9.33	10.33	13.67	19.67	21.00	31.33	65.00
Rhizobium radiobacter PCRE 10	12.67	14.33	15.67	17.67	26.00	56.00	84.00	73.00
Control	8.33	8.33	9.00	13.00	19.67	25.33	30.67	56.00
P. indica and *B. velezensis* PCSE 10	10.33	11.67	13.00	19.67	23.00	24.33	28.00	50.67
P. indica and *R. radiobacter* PCRE 10	14.00	16.33	18.33	30.00	34.00	54.67	69.67	86.00
SEm (±)	0.72	1.08	0.83	0.82	1.41	1.76	2.92	1.98
CD (0.05)	2.24	3.36	2.58	2.54	4.38	5.49	9.09	NS

*Mean of three replications having one plant per replication

Table 9. Leaf area per plant of bush pepper plants treated with various bioagents

Treatments	Leaf area (cm^2)/plant							
	Time of observation							
	1 MAT	2 MAT	3 MAT	4 MAT	5 MAT	6 MAT	7 MAT	8 MAT
Piriformospora indica	660. 00	704.66	1044.00	1650.00	2363.00	2474.33	2474.33	4035.66
Bacillus velezensis PCSE 10	464.00	610.33	604.33	884.66	1413.33	1518.33	1518.33	3245.83
Rhizobium radiobacter PCRE 10	788.33	831.00	764.33	1194.66	2200.00	3662.66	3662.67	3936.03
Control	515.00	509.33	647.66	1302.00	1163.83	1615.00	1615.00	2028.33
P. indica and *B. velezensis* PCSE 10	473.67	434.33	472.66	706.09	830.22	1681.50	1690.52	3002.46
P. indica and *R. radiobacter* PCRE 10	861.00	1006.67	1271.33	2257.00	2656.33	2442.33	2442.33	4586.33
SEm (±)	136.90	154.07	229.44	279.30	492.48	646.32	623.86	769.26
CD (0.05)	NS	NS	NS	860.67	NS	NS	NS	NS

*Mean of three replications having one plant per replication

Table 10. Number of spikes harvested per plant of bush pepper plants treated with various bioagents

Treatments	No. of spikes harvested/plant
Piriformospora indica	5.33±0.66
Bacillus velezensis PCSE 10	3.33±1.15
Rhizobium radiobacter PCRE 10	5.00±0.66
Control	2.00±0.66
P. indica and *B. velezensis* PCSE 10	4.66±1.20
P. indica and *R. radiobacter* PCRE 10	6.00±0.66
SEm (±)	1.81
CD (0.05)	NS

*Mean (± SD) of three replications having one plant per replications

Table 11. Mean spike length per plant of bush pepper plants treated with various bioagents

Treatments	Mean spike length(cm)
Piriformospora indica	11.38 ± 0.66^{a}
Bacillus velezensis PCSE 10	12.26 ± 0.43^{a}
Rhizobium radiobacter PCRE 10	12.02 ± 0.81^{a}
Control	6.65 ± 0.27^{c}
P. indica and *B. velezensis* PCSE 10	10.89 ± 1.15^{ab}
P. indica and *R. radiobacter* PCRE 10	8.44 ± 0.61^{bc}
SEm (±)	0.72
CD (0.05)	2.58

*Mean (± SD) of three replications. Figures in a column followed by the same alphabet do not differ significantly according to DMRT ($P \leq 0.05$).

4.2.1.6. Fresh and dry weight of berries

Observations in dry weight and fresh weight of berries were taken after harvesting the spikes in the 8th month after transplanting (Table 12). When the berry fresh weight as well as dry weight were analyzed statistically, there were no significant difference observed among the treatments. The highest value was recorded in the treatment involving single application of *P. indica*.

4.2.1.7. Root Colonization by Piriformospora indica

The extent of root colonization by the fungal entophyte was assessed by staining the roots of all the surviving plants treated with *Piriformospora indica* and the combinations of *Piriformospora indica* with bacterial isolates (Table 13). Pear shaped chlamydospores were observed under 40 X magnification in a compound bright field microscope within the cortical cells of root tissues of the inoculated bush pepper plants when detection was carried out at one month after transplanting. No colonization was observed in the treatments including uninoculated control plants and in individual bacterial application.

The percentage root colonization of *P. indica* is given in Table 18. Plants treated with *Piriformospora indica* alone showed highest root colonization with 35.50 percent followed by a combination by *P. indica* and *Bacillus velezensis* (31.11 per cent), *P. indica* and *Rhizobium radiobacter* PCRE10 (19.35 percent).

4.4.2. Disease suppression in the established bush pepper plants

4.4.2.1. Lesion Size on inoculated leaves

Lesion size was recorded at daily intervals after challenge inoculation with the pathogen. The data thus obtained were further processed for finding Disease Index (DI). Observation on disease incidence from fourth and seventh day was statistically analyzed. Lesion size showed significant difference at four days after inoculation, whereas observations after seven days of inoculation were non-significant (Table 14)

Table 12. Fresh and dry weight of berries per plant of bush pepper plants treated with various bioagents

Treatments	Fresh weight of berries(g)	Dry weight of berries(g)
Piriformospora indica	5.71 ± 0.74	2.13 ± 0.21
Bacillus velezensis PCSE 10	5.26 ± 0.76	1.64 ± 0.19
Rhizobium radiobacter PCRE 10	2.58 ± 0.10	0.75 ± 0.06
Control	3.00 ± 1.25	0.93 ± 0.44
P. indica and *B. velezensis* PCSE 10	3.61 ± 0.79	1.25 ± 0.36
P. indica and *R. radiobacter* PCRE 10	3.92 ± 1.87	1.28 ± 0.66
CD (0.05)	NS	NS

*Mean (± SD) of three replications having one plant per replication.

Table 13. *P. indica* root colonization (%) in bush pepper (one month after transplanting)

Treatments	*P. indica* root colonization (%)
Piriformospora indica	35.5
P. indica and *Bacillus velezensis* PCSE 10	31.11
P. indica and *Rhizobium radiobacter* PCRE 10	19.35

* Observation from 100 root bits

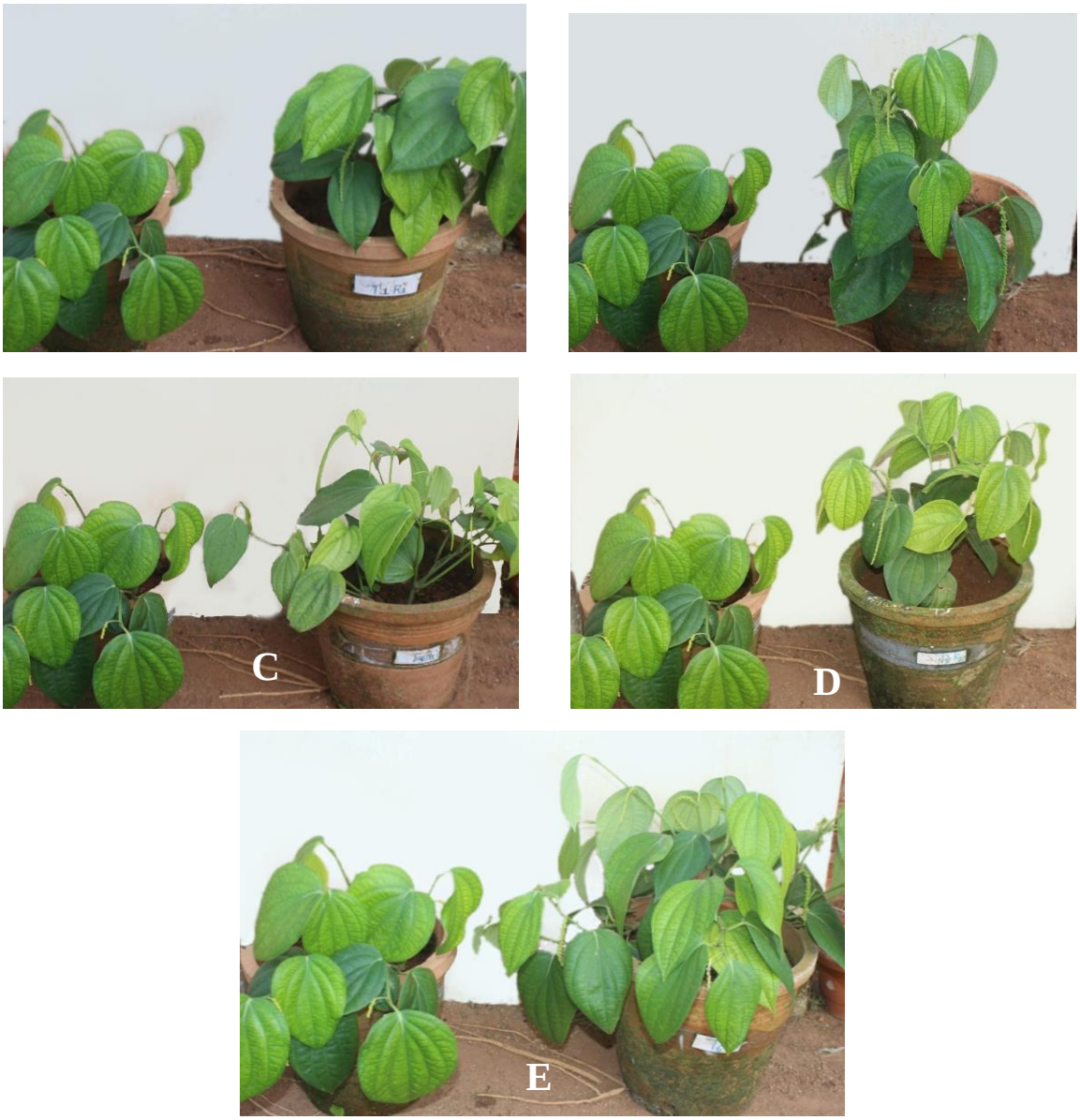


Plate 11. Assesesment of plant growth promotion by bioagents in black pepper variety Panniyur- 1

A- Control VS *Piriformospora indica*; B-Control VS *B. velezensis* PCSE 10; C-Control VS *R. radiobacter* PCRE 10 ; D- Control VS Combination of *P. indica* and *B. velezensis* PCSE-10; E-Control VS Combination of *P. indica* and *R. radiobacter* PCRE 10

Seven days after inoculation, the minimum disease incidence was noticed in the plants treated with combination of *P. indica* and *Rhizobium radiobacter* PCRE10 (0.43 cm).

4.4.2.2. Disease index

Treatments had no significant effect on the disease index calculated with the lesion size on inoculated leaves at four and seven days after inoculation. The lowest disease index was observed in plants treated with combination of *P. indica* and *Rhizobium radiobacter* PCRE10 (0.20). This was followed by plants treated with *Bacillus velezensis* PCSE10 and its combination with *P. indica* with a disease index of 0.22(Table15).

Table 14. Leison size on inoculated leaves

Treatments	Leison size (cm)	
	4 DAI	**7 DAI**
Piriformospora indica	0.78 ± 0.30 [a]	2.96 ± 0.89 [a]
Bacillus velezensis PCSE 10	0.13 ± 0.06 [b]	0.99 ± 0.65 [b]
Rhizobium radiobacter PCRE 10	0.08 ± 0.04 [b]	1.41± 0.55 [b]
P. indica and *B. velezensis* PCSE 10	0.09 ± 0.01 [b]	0.88 ± 0.58 [b]
P. indica and *R. radiobacter* PCRE 10	0.03 ± 0.01 [b]	0.44 ± 0.42 [b]
Pathogen control	0.01 ± 0.06 [b]	0.48 ± 0.35 [b]
Chemical control	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]
Absolute control	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]
CD (0.05)	0.11	1.471

*Mean (± SD) of five replications having two leaves per replication. Figures in a column followed by the same alphabet do not differ significantly according to DMRT (P≤ 0.05).
DAI: Days after inoculation

Table 15. Disease index after seven days of inoculation

Treatments	Disease index
Piriformospora indica	0.62 ± 0.08 [a]
Bacillus velezensis PCSE 10	0.22 ± 0.13[bc]
Rhizobium radiobacter PCRE 10	0.45 ± 0.11 [ab]
P. indica and *B. velezensis* PCSE 10	0.22 ± 0.13[bc]
P. indica and *R. radiobacter* PCRE 10	0.20 ± 0.12[bc]
Pathogen control	0.30 ± 0.09[abc]
Chemical control	0 ± 0.00 [c]
Absolute control	0 ± 0.00 [c]
CD (0.05)	0.34

*Mean (± SD) of five replications having one plant per replication. Figures in a column followed by the same alphabet do not differ significantly according to DMRT (P≤ 0.05).
DAI: Days after inoculation

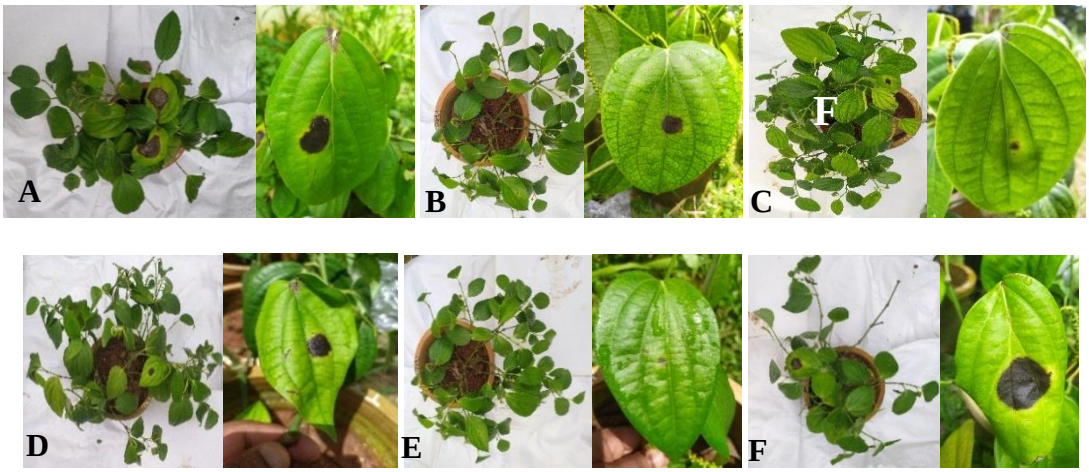


Plate 12 Bush pepper plants treated with the endophytes showing foliar symptoms of infection six days after challenge inoculation with *Phytophthora capsici*. Representative leaves from the corresponding infected plants are also shown. A- *Piriformospora indica*, B- *Bacillus velezensis* PCSE 10, C- *Rhizobium radiobacter* PCRE 10, D- *P. indica* and *Bacillus velezensis* PCSE 10, E- *P. indica* and *Rhizobium radiobacter* PCRE 10, F- Pathogen control

Discussion

5. DISCUSSION

The productivity of farming systems has been improved due to the use of chemical pesticides and fertilizers which are used for controlling pests and supplying essential nutrients for plant growth. Hazell and Wood (2008) reported the negative effects of long-term use of chemical products in field of agriculture which negatively affect the soil quality by reducing the yielding capacity, pollutes the underground water and destroys biodiversity. Also, the continuous use of fertilizers affects public health. Nowadays there is growing concern among public regarding the after effects of long-term use of agrochemicals. High input agriculture production methods and indiscriminate use of pesticides and fertilizers resulted in decline in plant resistance to abiotic and biotic stress factors. Use of microorganisms which benefits plants contributes positively to sustainable agriculture is an environmentally safe alternative for agrochemicals (Vessey, 2003). The application of bioagents are helpful in agriculture. This includes plant growth promotion, biological disease control, yield increases, and quality upgradation. Studies are conducted in recent years to develop formulated products of biofertilizers and biocontrol agents as alternatives to chemical fertilizers and pesticides (Gray and Smith, 2005; Singh *et al*., 2011). Beneficial microbial inoculants used in agriculture includes plant growth-promoting bacteria and fungi. According to their functions they are grouped in biofertilizers and biocontrol agents. According to Malusa and Vassilev (2014) biofertilizers and biocontrol agents are formulated products containing one or more microorganisms that enhance the nutrient status and health of the plants by either replacing soil nutrients and/or by making nutrients more available to plants and/or by increasing plant access to nutrients or by producing specific metabolites. Major constraints in development of formulated products are the difficulties related with production and formulation of inocula. The biofertilizer development starts with isolation, selection, and characterization of an essential microorganism. The process ends with fermentation mass production process and formulation procedure which is considered as the major technological steps involved in the development (Vassilev *et al.*, 2015; 2016).

Use of bioagents are the safe and potential alternative for chemical fertilizer and pesticides. Extensive use of chemical fertilizers and pesticides are hazardous to environment and human beings and are not economical to farmers. Numerous studies are being conducted all around the world to explore the potential of the microorganisms as an alternative for agrochemicals. Nowadays, different bioagents like rhizosphere microorganisms, AMF, endophytes etc. are gaining importance. A consortium of beneficial microorganisms improves the efficiency of a bio-inoculant formulation, by improving the consistency of its performance.

Mycorrhizal fungi and plant-growth promoting microorganisms (*Rhizobium*, Phosphorus solubilizing microorganisms, etc.) were found to play an important role in plant growth. They manifest various mechanisms in plants to obtain final result (Vassilev *et al.,* 2017). During last decades several developments took place in the field of development of microbial formulations. *Piriformospora indica* which belonging to Basidiomycetes were found to exert different functions. This includes plant growth promotion and Phosphorus solubilization. This fungus can grow in axenic conditions and are easy to handle. Mass multiplication of this fungus is possible in conventional fermentation systems. When culture filtrate of *P. indica* was introduced into soil- water systems demonstrated with same effects as spores/mycelium (Bagde *et al*., 2011; Kumar *et al*., 2012).

Foot rot diseases of black pepper is caused by pathogen *Phytophthora capsici*, is one of the major problems faced by black pepper growers of Kerala, especially during the southwest monsoon season in the nursery (Sarma *et al*., 1994). The black pepper vine at any of its growth stage is susceptible to this pathogen and infect all parts of the plant i.e. root, collar, leaves, stem, berries and inflorescence. Cultural practices including Phyto-sanitation and repeated prophylactic application of copper fungicides are the recommended measures for the management of this disease. Several reports regarding the use of microbial antagonists for the management of foot rot disease of black pepper

(Anith and Manomohandas, 2001, Anith *et al.,* 2002: Rajan *et al.,* 2002: Shobha and Murthy, 2018).

The results from the study conducted by Jasim *et al.* (2013) confirms the promising applications of the endophytic bacterial isolates as growth promoters in *P. nigrum*. The current research focused on the usage of bacterial endophytes, the endophytic fungus *P. indica* and their combination as growth promoter and biocontrol agents in controlling the foot rot disease in black pepper.

Senthilkumar *et al.* (2011) stated that the intimate association of bacterial endophytes with plants offers a unique opportunity for their potential application in plant protection and biological control. The bacterial endophytes used in the study *Bacillus velezensis* PCSE10 and *Rhizobium radiobacter* PCRE10 were isolated from the exotic pepper variety *Piper colubrinum* from the stem and root respectively. They had plant growth promotional activity in black pepper plantlets and biocontrol activity against the foot rot pathogen *Phytophthora capsici* (Kollakkodan, 2017; Kollakkodan *et al*., 2017).

The bacterial endophytes used in the present study are endophytic bacteria from *Piper colubrinum* which have more innate potential in suppressing the foliar infection caused by *Phytophthora capsici*. *Piper colubrinum* is highly resistant to *P. capsici* infection and is used as root stock for production of bush pepper by farmers. This exotic pepper variety has immense potential as a donor plant in breeding programs for the improvement of the cultivated species, *P. nigrum* (Dicto and Manjula, 2005). Among all isolates which Kollakkodan (2017) isolated, endophytic bacteria from *Piper colubrinum*, showed more antagonistic activity against *P. capsici*. Further study should be conducted in order to ascertain the reason behind the improved resistance of *Piper colubrinum* against *P. capsici* infection and role of endophyte in disease suppression.

The fungal root endophyte used in the study was *Piriformospora indica*. *P. indica* is an AM like fungus, originally isolated from the roots of xerophytic woody shrubs in the Thar Desert in India (Verma *et al.,* 1998; Varma *et al.,* 1999). As reported by several

authors it interacts beneficially with a wide range of plants and is known to enhance plant growth, biomass production, phosphorus acquisition and act as a bio-protector against abiotic and biotic stress including root and leaf fungal pathogens (Serfling *et al.,* 2007; Wang *et al*., 2015; Anith *et al*., 2018). Exploitation of endophyte–plant interactions can play a significant role in low-input sustainable agriculture applications for both food and nonfood crops and can result in the promotion of plant health.

The whole course of the research was divided into two, *in vitro* and *in vivo* experiments. As a preliminary step to initiate the study, isolation of virulent isolate of *P. capsici* from the leaves of the diseased plants showing lesion symptoms was performed by placing leaf bits on half plates of potato dextrose agar media incorporated antibiotic cephalexin. The medium suppressed the growth of bacteria in the plates pure culture of *P. capsici* devoid of bacterial contamination was obtained. Many researchers have isolated *P. capsici* in a similar way from infected leaves by adding suitable antibiotic solutions (Anith *et al*., 2002; 2003, Ozgonen and Erkilic, 2007; Kollakkodan, 2017).

Assessment of *in vitro* antagonism between the endophytic fungus and the bacterial bioagents is one of the most important pre-requisites to assess their compatibility in growth promotion and biocontrol strategy. *In vitro* trials are preliminary screening methods, which can be used to choose the combination of biological agents for efficient consortium development (Anith *et al*., 2003; Lemessa and Zeller, 2007). *In vitro* evaluation of antagonism of bacteria against the endophytes was performed by dual culture plate method (Dennis and Webster, 1971; Li *et al*., 2002; Anith *et al*., 2003; Aravind *et al*., 2009). Combination of organisms to be used in the *in vivo* experiment would be selected by this assay. Though the laboratory conditions and field conditions vary, it is assumed that the results obtained in the lab condition may get reflected in the field trial as well.

In the present study, antagonism against *P. indica* on PDA media by the dual culture plate method was done to know about the *in vitro* interaction. Compatibility was assessed by lack of any inhibition zone when *P. indica* and bacterial isolates cultured

together in PDA, whereas, the non-compatible ones would develop zone of inhibition. In the present screening on PDA plates *Bacillus velezensis* PCSE10 was found to be incompatible with *P. indica* as it developed zone of inhibition whereas *Rhizobium radiobacter* PCRE10 was compatible with the fungus. Incompatible reaction suggests that there may be an inhibition in the growth of *P. indica* by the metabolites secreted by the endophytic bacteria when applied together in the rhizosphere. Dual culture plate assay done by Athira (2018) using the same bacterial isolates with *P. indica* on PDA exhibited same type of interaction pattern which was similar to that of the result obtained in the present study using the same isolates.

Mixed inoculation of different biological agents on crop plants is not advisable when there is antagonism among them. However, incompatibility of the inoculants could be resolved by following a temporal separation in application to the root zone of the crop plants (Anith *et al.,* 2011). Antagonistic reaction against *P. indica* by *Trichoderma harzianum* was proven. It can even use as dual inoculants in tissue cultured black pepper, if *P. indica* was applied at an early hardening stage, followed by the application of *T. harzianum* during transplanting to the field.

In vitro antagonism against *P. capsici* by bacterial and fungal endophyte indicated by the presence of an inhibition zone in dual culture plate assay. The bio agents which failed to exhibit any inhibition on mycelial growth of *P. capsici* in dual-culture inhibition tests indicate that the bioagents do not produce any antibiotic metabolites when grown on culture plates. Several authors have been reported that competition, antibiosis and mycoparasitism were the biocontrol traits antagonist conferring for *in vitro* inhibition against plant pathogens (Narisawa, *et al*., 2004; Bailey *et al*., 2008; Morath *et al*., 2012; Sreeja *et al*., 2016). The production of inhibitory substance may be the reason for *in vitro* inhibition of these isolates against *P. capsici*. However, results of dual culture assay may contrast with the *in vivo* performance were reported earlier by several authors (Baker, 1968; Schroth and Hancock, 1981; Wong and Baker, 1984; Anith *et al.,* 2003).

Detached leaf assay produced lesions of varying sizes on leaves. It was analyzed by measuring the lesion size in centimeters taken four days after pathogen inoculation. Anith, *et al*. (2003) stated as there was no involvement of host in dual culture technique, an assay involving interaction of the pathogen, antagonist, and the host plant, which better resembled the field conditions is expected to convey a rigid basis for antagonism.

There was difference in the time taken for appearance of lesion on treated leaves. In control and in leaves treated with many of the plant associated bacteria of *P. nigrum*, the disease was observed within 30 to 38 hours (Kollakkodan, 2017). However, in leaves treated with *P. colubrinum* bacterial isolates PCRE10 and PCSE10 it took 48 hours for disease development. This observation ascertains the fact that *P. colubrinum* associated bacteria possess certain resistance characters against *P. capsici* infection. *P. colubrimum* has attained great importance due to its resistance towards *P. capsici* (Ravindran and Remashree, 1998).

Piriformospora indica improves defensive capacity of several crop plants against fungal, nematode and viral pathogens (Lakshmipriya *et al*., 2017; Varkey *et al*., 2018; Ashar, 2019; Chandan, 2019). Ghorbanpour *et al*. (2018) reported that the protective effect of different beneficial fungi can result from various mechanisms directed against pathogens, such as competition for nutrition, antibiosis, mycoparasitism and induction of plant systemic resistance.

According to most authors, *P. indica* more likely it protects plants by inducing their systemic immunity (Serfling *et al.,* 2007; Oelmuller *et al.,* 2009; Molitor *et al.,* 2011; Pedrotti *et al.,* 2013; Johnson *et al.,* 2014; Narayan *et al.,* 2017).

In the detached leaf assay, minimum lesion size was observed in leaves treated with *Rhizobium radiobacter* PCRE10 with a lesion size of 1.43 cm which caused 52.33 % disease suppression over control. *Bacillus velezensis* PCSE10 with a lesion size of 2.18 cm which caused 27.33 per cent reduction in disease. The root endophyte from *P. colubrinum* pioneered in reducing the lesion size on inoculated leaves. The reduction in

the lesion size indicate that the isolates have successfully suppressed the disease due to direct antagonism. In the detached leaf assay performed by Melnick *et al*. (2008), it was reported that *Bacillus cereus* BT8 induced resistance against *P. capsici* in cacao.

The bacterial isolates were tested for the production of plant growth promoting and disease suppressing metabolites like IAA and ammonia. Both the isolates were found to produce IAA. Indole Acetic Acid (IAA) is considered to be the most important native auxin. IAA belongs to the group of phytohormones (Strzelczyk and Pokojska, 1984). Endophytic bacteria within the plant release auxin which results in the formation of lateral, adventitious roots and root hairs thereby increasing the root surface area thus promote plant growth (Hilbert *et al*., 2012). *Rhizobium radiibacter* PCRE 10 was found to produce 4.616 µg/ml and 8.793 µg/ ml IAA without and with L- Trypthophan respectively. *Bacillus velezensis* PCSE 10 was found to produce 3.768 µg/ml and 4.920 µg/ml IAA in absence and with L- Tryptophan respectively. As both the bacterial isolates were produced IAA in absence of tryptophan of demonstrating tryptophan independent IAA synthesis by *R. radiobacter* PCRE 10 and *Bacillus velezensis* PCSE 10.

Yingwu *et al*. (2009) reported that some endophytic microorganisms upon colonization on plants increase growth which attributed to their potential to synthesize IAA. Tryptophan is used as the precursor by microbes in the biosynthesis of IAA via tryptophan dependent route. Bacterial IAA in conjugation with endogenous auxins produced by host plant stimulates promotion of growth.

Bianco *et al*. (2006) stated that IAA production by endophytic bacteria may increase the growth of the host plants. IAA produced by bacteria can provide protection against stresses by coordinately improving different cellular defense systems. Several authors reported the detection of plant growth promoting beneficial microorganisms can be detected by screening based on IAA production (Ali and Hasnain, 2007; Govindarajan *et al*., 2007; Jasim *et al*.,2013). Although IAA production is widespread among many plants associated bacteria, studies on such an aspect among endophytic isolates from *P. colubrinum* is very limited. As a plant with much biotechnological importance as a source

of resistance and defense genes could be used as a source to confer resistance to a variety of plant pathogens infecting spice crops, identification of microorganisms with growth promoting effect from *P. colubrinum* is very important. This highlights the novelty, significance and promising applications of the study.

Jasim *et al.* (2013) have screened seven bacterial isolates from *P. nigrum* by based on their ability to produce IAA. Prasad and Dagar (2014) reported IAA production by endophytes isolated from Avocado and grapes with maximum production by strain SA 3.

The most important trait of PGPR is the production of ammonia that indirectly influences the plant growth. In the present study *Rhizobium radiobacter* PCRE10 was found positive for ammonia production.

Ammonia production is an important trait of PGPR which influences plant growth indirectly. An amino acid tryptophan is hydrolyzed into indole, pyruvic acid, and ammonia by enzyme tryptophanase which is present in bacteria. Pyruvic acid and ammonia are used by bacteria to satisfy its nutritional needs (Prescott and Harley, 2002). Incorporation of ammonia nitrogen into organic material relatively easy and direct. Because as compared to other forms of inorganic nitrogen, ammonia nitrogen is more reduced. In the Rhizobium legume interaction, the ammonia in the bacterial cell diffuses out and is assimilated in the legume cell (Prescott and Harley, 2002).

Present study was conducted by adopting “bush pepper”, a system of miniaturized plants raised from plagiotrops of black pepper plants that made the observation on yield of the crop within one year. In the current study, variety of black pepper, Panniyur-1 was used to evaluate the efficiency of two bacterial bioagents and the fungal endophyte *P. indica*, each individually and in combination with the fungus in suppressing the foot rot pathogen incidence.

Rooting of plagiotrops was found to be less as compared to runner shoots. One of the major steps in the production of bush pepper is standardization of suitable rooting

conditions. Mol *et al.* (2017) conducted a study to investigate the rooting and establishment of laterals. Coir pith compost was incorporated in the potting mixture and the plants were kept under different humidity conditions. When plagiotropic branches of pepper variety, Panniyur - 1 were planted in 4'' x 6'' polyethylene bags and kept under 50% shade for rooting, a success rate was found 35percent (Lakshmana *et al.,* 2016). On a study conducted in one of the farmer's nursery success rate of rooting and sprouting of lateral branches were varied from 10-40% (Prakash *et al.,* 2016). It is evident that the establishment rate of rooting obtained during the present study is similar to that of previous studies. In the present study combination of bacterial endophytes with *P. indica* reduced establishment as compared to their individual application (Fig 1).

Plants inoculated with *P. indica* in combination with *Rhizobium radiobacter* PCRE 10 exhibited significant improvement in major growth parameters. The mean number of leaves per plant (Fig 2) and the leaf area per plant (Fig 3) in the inoculated treatment recorded an increase compared to that of the control plants starting from the first month of transplanting. Yield characteristics such as the number of berries per spike (Fig 4) and mean spike length (Fig 5) though statistically non-significant, were more profound in the case of the endophyte treated plants compared to that in the un-inoculated control. *P. indica* has been reported to have some close association with other bacterial flora. These intimate bacterial species that are present within the hyphae of the fungus included a *Rhizobium radiobacter* strain as well (Sharma *et al.,* 2008). *Rhizobium radiobacter* PCRE 10 has been reported to be an efficient plant growth promoter and biocontrol bacterium in black pepper also (Kollakkodan, 2017).

Plants treated with *Piriformospora indica* showed highest root colonization followed by combination by *P. indica* with *Bacillus velezensis* PCSE 10 and combination of *P.indica* and *Rhizobium radiobacter* PCRE 10 (Fig 6). Anith *et al.* (2011) reported positive effect on vegetative growth in tissue cultured black pepper on colonization of *P. indica*. Persistent nature of colonization on bush pepper plants after six months of inoculation is evidenced by the detection of chlamydospores (Anith *et al.,* 2018). In the present study, individual application of fungal bioagent showed maximum root

colonization (35.50 %) when root colonization pattern was analyzed one month after transplanting.

In the present study the treatments that involved combination with bacterial bioagents, reduced colonization was observed. Bacterial bioagents may have some adverse influence on the fungal root colonization, though the same has not been completely prevented. Varkey (2018) reported a reduction in the amount of root colonization by *P. indica* in tomato plants when combined inoculation was done along with *B. pumilus* VLY 17 and *P. fluorescens* AMB 8. A reduction in the amount of root colonization by *P. indica* in tomato plants when combined inoculation was done with *Rhizobium radiobacter* PCRE 10 and *Bacillus velezensis* PCSE 10 has already been reported (Athira, 2018). A rise in the amount of root colonization by *P. indica* in chilli plants when cocultured with *Pseudomonas fluorescens* PN026 has already been reported (Nandana, 2019).

The fundamental role of any biocontrol agent against plant diseases is to suppress or inhibit the development of disease in the field conditions. As a course to evaluate the efficacy of the bacterial and fungal endophytes, a pot culture experiment was carried out. Black pepper variety Panniyur-1 was used for assessing the biocontrol potential of the endophytes. The favourable end result of the use of biocontrol agents against any soil-borne pathogens depends greatly on the activity of the isolates on the host plant, which in turn depends on the delivery of the isolates. Methods of delivering biocontrol agents onto host plants were suggested by various authors which includes seed treatment (Islam *et al.*, 2016), bacterization of propagating plant material (Aravind *et al*., 2012; Kollakkodan *et al*., 2017), foliar spray and soil application (Bressan and Borges, 2004). Endophytic bacteria can be delivered into the plant system prior to planting of cuttings in nursery. In the experiment to study the disease suppression in bush pepper, endophytes were applied as bacterization of cuttings and also during transplanting into larger pots.

On the fourth day after inoculation, before the leaves fell off, the lesion sizes were recorded and compared. The lowest lesion size was observed in plants treated with the

combination of *P. indica* and *Rhizobium radiobacter* PCRE10 with a lesion size of 1.18 cm causing 75.05% disease suppression over the pathogen control. Highest lesion size was observed in *P. indica* treated plants (4.73 cm).

Various mechanisms used by rhizobia in disease control include production of antibiotics, siderophores, and phytoalexins (Chakraborty and Purkayastha, 1984; Chakraborty and Chakraborty, 1989; Guerinot *et al*., 1990). Tu (1978) reported that *Rhizobium* reduced root rot of soyabean caused by *Phytophthora megasperma*. Antgonistic rhizobia and bradyrhizobia, when used as seed dressing and soil drench significantly reduced the fungal dieases in both legumes and non-legumes (Enteshamul-Haque and Ghaffer, 1993).

Foot rot disease caused by *P. capsici* was the most destructive and devastating one among the various diseases affecting black pepper. The disease continues its prevalence, although several chemical and biological methods are in use, which necessitates a re-orientation in its management strategy. The information generated in the present study about endophytic bacteria from *Piper colubrinum* and root endophytic fungus *P. indica* will be helpful in evolving better management strategies having greater biocontrol potential. Endophytes obtained from the wild exotic species *P colubrinum* showing greater benefits in terms of both disease suppression and plant growth promotion paves a way for developing potential antagonists that could circumvent the deleterious effects of the infection caused by the pathogen.

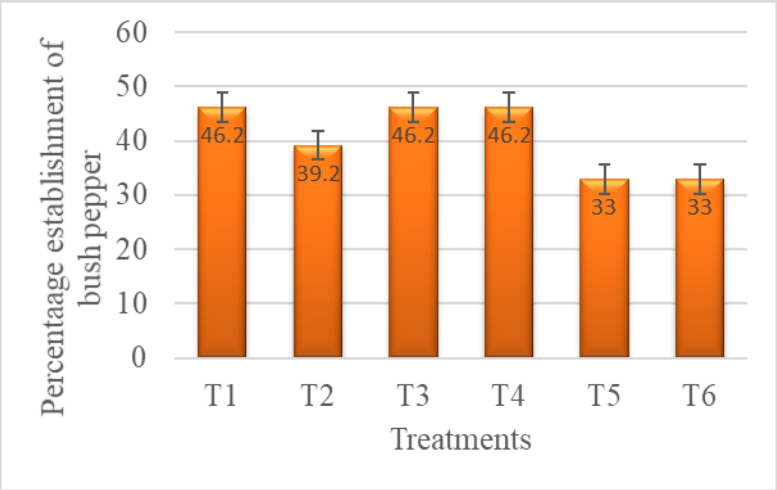


Figure 1: Percentage of establishment of bush pepper

T1- *P. indica*; T2- *Bacillus velezensis* PCSE 10; T3- *Rhizobium radiobacter* PCRE 10; T4- Control; T5- Combination of *P. indica* and *Bacillus velezensis* PCSE 10; T6- Combination of *P. indica* and *Rhizobium radiobacter* PCRE 10

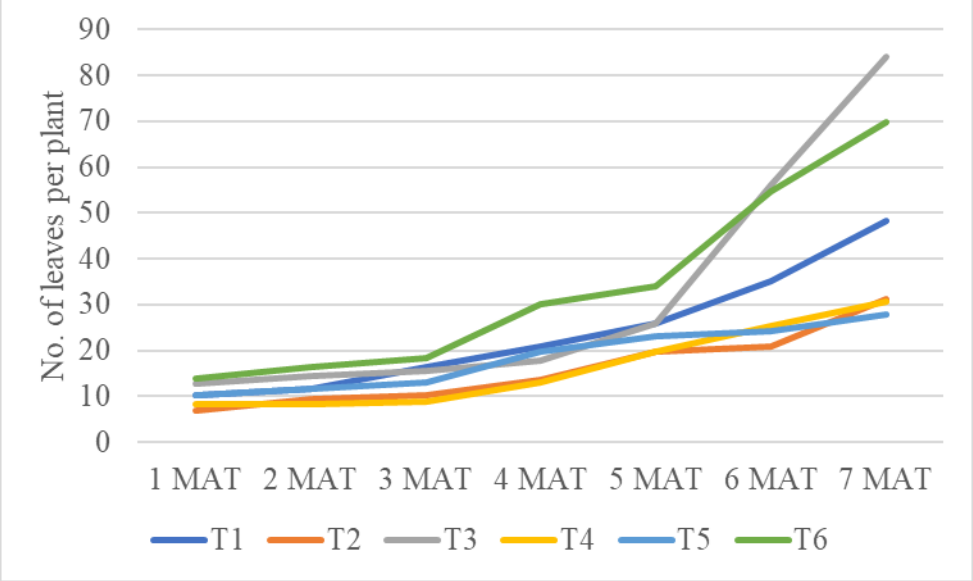


Figure 2: Number of leaves of the black pepper variety Panniyur-1 taken in one month interval on inoculation with bioagents

T1- *P. indica*; T2- *Bacillus velezensis* PCSE 10; T3- *Rhizobium radiobacter* PCRE 10; T4- Control; T5- Combination of *P. indica* and *Bacillus velezensis* PCSE 10; T6- Combination of *P. indica* and *Rhizobium radiobacter* PCRE 10

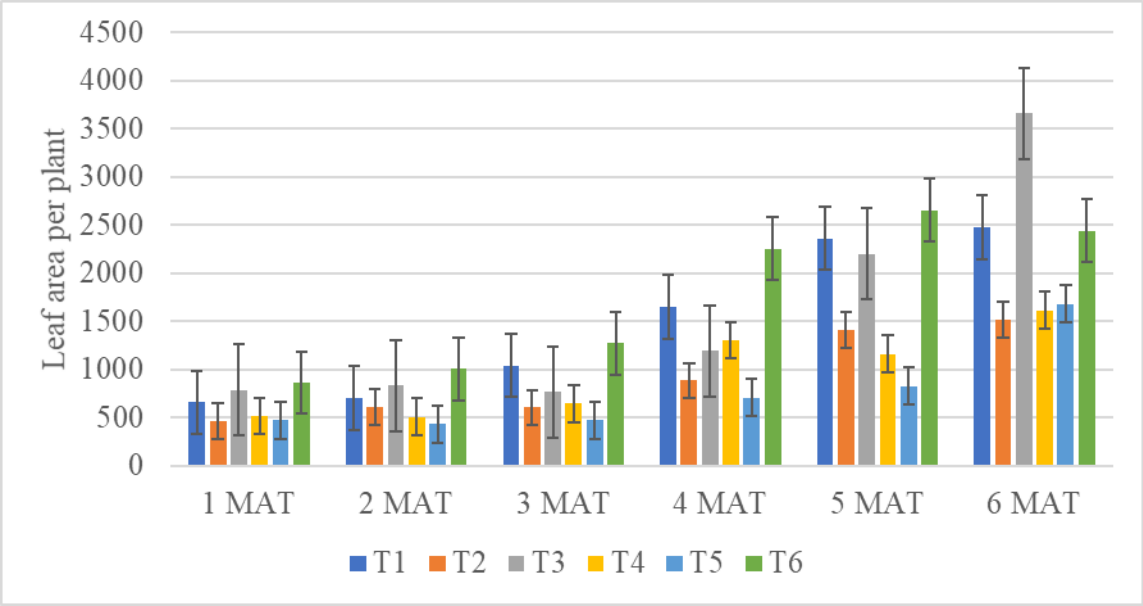


Figure 3: Leaf area (cm^2) per plant of the black pepper variety Panniyur-1 taken in one month interval on inoculation with bioagents

T1- *P. indica*; T2- *Bacillus velezensis* PCSE 10; T3- *Rhizobium radiobacter* PCRE 10; T4- Control; T5- Combination of *P. indica* and *Bacillus velezensis* PCSE 10; T6- Combination of *P. indica* and *Rhizobium radiobacter* PCRE 10

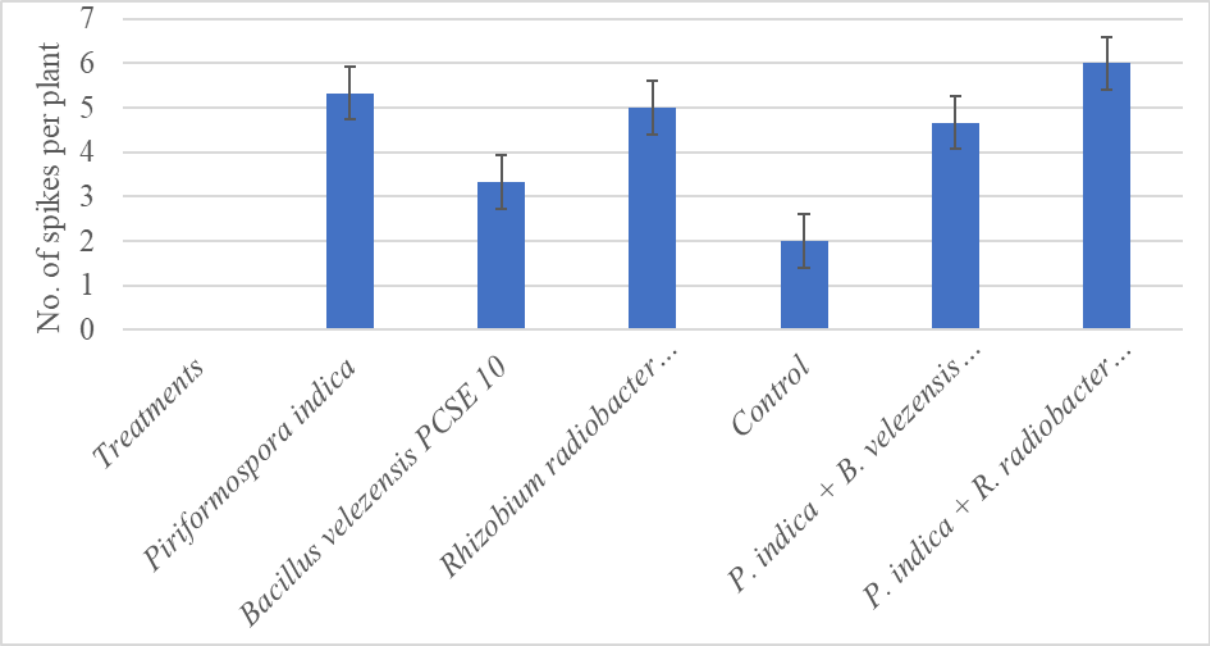


Figure 4: Number of spikes harvested per plant of the black pepper variety Panniyur-1 taken in one month interval on inoculation with bioagent

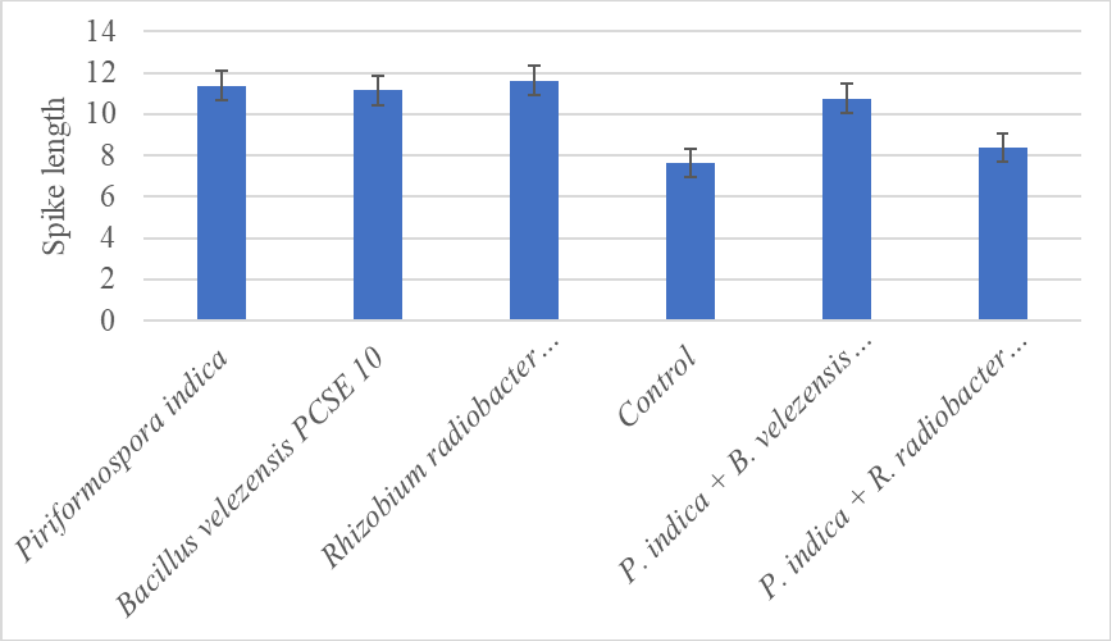


Figure 5: Spike length of the black pepper variety Panniyur-1 taken in one month interval on inoculation with bioagents

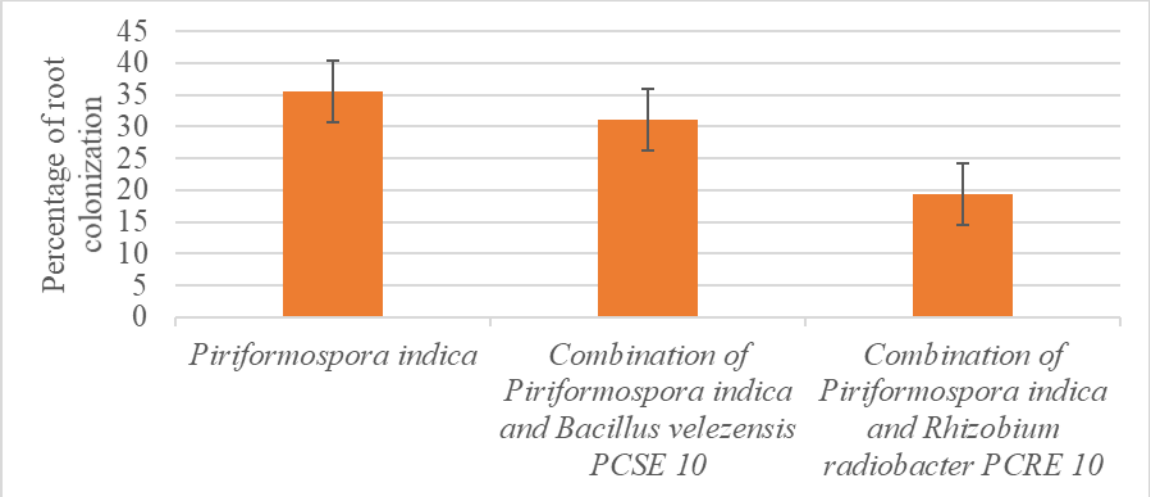


Figure 6: Root colonization in percentage of the black pepper variety Panniyur-1 on inoculation with bioagents

Summary

6. SUMMARY

Foot rot diseases of black pepper is caused by pathogen *Phytophthora capsici,* is one of the major problems faced by black pepper growers of Kerala, especially during the southwest monsoon season in the nursery (Sarma *et al*., 1994). The black pepper vine at any of its growth stage is susceptible to this pathogen and infect all parts of the plant i.e. root, collar, leaves, stem, berries and inflorescence. Cultural practices including Phyto-sanitation and repeated prophylactic application of copper fungicides are the recommended measures for the management of this disease. Several reports regarding the use of microbial antagonists for the management of foot rot disease of black pepper (Anith and Manomohandas, 2001, Anith *et al.,* 2002: Rajan *et al.,* 2002). The increased use of hazardous fungicides in the field of agriculture in recent years has been subject to discussion of conservationists and public health workers. Use of microbes which can be utilized as biocontrol agents and also helps in plant growth and yield is a safe alternative, as this method demands low amounts of chemical inputs to farming systems, which in turn results in reduction in cost of pest and disease control measures. This also reduces pollution hazards, with less disturbances of biological equilibrium (Senthilkumar *et al.,* 2011).

In this context, a study entitled "Plant growth promotion and foot rot disease suppression in black pepper using fungal and bacterial endophytes*"* was conducted during 2018-2020 at Department of Agricultural microbiology, College of Agriculture, Vellayani. Major objective of the study was to assessand evaluate the compatibility of the root endophytic fungus *Piriformospora indica* and two endophytic bacterial strains *Bacillus velezensis* PCSE 10 and *Rhizobium radiobacter* PCRE 10 and their effect on growth promotion in bush pepper and foot rot disease suppression in black pepper.

The salient findings of the present study are as follows.

In vitro interaction of bacterial bioagents and *Piriformospora indica* using dual culture plate assay was done to assess the compatibility between them. The zone of

inhibition was measured in 6th day after the bacterial inoculation. In PDA plates *Bacillus velezensis* PCSE10 inhibited the growth of *Piriformospora indica* with an inhibition of 4 mm in 6th day after bacterial inoculation.

Virulent isolate of foot rot pathogen *Phytophthora capsici* were isolated from infected black pepper leaves from the fields of College of Agriculture, Vellayani on PDA medium. The pathogenicity of the strain was proved by artificial inoculation on black pepper leaves.

In vitro antagonism shown by the bacterial bioagents against the pathogen was evaluated using dual culture plate assay. All the three bioagents were found to have *in vitro* antagonism against *Phytophthora capsici.* In the dual culture plate assay, largest zone of inhibition was produced by *Bacillus velezensis* PCSE10 (4.67 mm) and *Rhizobium radiobacter* PCRE 10 (1.03 mm).

The dual culture plate assay was followed by a detached leaf assay using the two endophytic bacterial isolates. There was significant difference in lesion size on control leaves sprayed with sterile water and leaves sprayed with the selected isolates. The minimum lesion size was observed in leaves treated with *Rhizobium radiobacter* PCRE10 with a lesion size of 1.43 cm which caused 52.33 % disease suppression over control which was on par with leaves treated with *Bacillus velezensis* PCSE 10 with lesion size of 2.18 cm and caused 27.33% disease suppression over control. The maximum lesion size was observed in control leaves sprayed with distilled water (3.0 cm).

All the isolates produced significant quantity of IAA under *in vitro* conditions. *Rhizobium radiibacter* PCRE 10 was found to produce 4.62 µg/ml and 8.79 µg/ ml IAA without and with L- Trypthophan respectively. *Bacillus velezensis* PCSE 10 was found to produce 3.77 µg/ml and 4.92 µg/ml IAA in absence and with L- Tryptophan respectively.

Among the plant growth promoting traits screened, it was observed that isolate *Rhizobium radiobacter* PCRE 10 was positive for Ammonia as noted by the development of brown to yellow colour.

In vivo pot culture experiments were conducted using individual as well as combined application of the bioagents in unsterile soil. Black pepper stem cuttings were initially raised in polythene bags filled with sterile sand, soil and FYM (1:1:1 ratio) as planting medium. Bacterization of the cuttings was done with 48 h old culture of bacterial bioagent, 20 minutes prior to planting. Mycelium of *Piriformospora indica* mixed with sterile vermiculite was incorporated into the planting holes at the rate of one per cent (w/v). After imposing the treatments, cuttings were planted into the polythene bags. After four months, rooted cuttings were transplanted to earthen pots filled with garden soil and FYM. Bacterial inoculation was done in the roots by pouring 100 ml bacterial suspension in root zone. Mycelium of *P. indica* mixed with sterile vermiculite was incorporated into the planting holes at the rate of one per cent (w/v). Challenge inoculation of the pathogen was done eight months after transplanting and daily observations were recorded for three weeks.

Highest number of leaves were recorded in the plants treated either with single or combination of *P. indica* and *Rhizobium radiobacter* PCRE 10. In the observation taken at first month after transplanting higher number of leaves (14) was recorded in the treatment with combined application of *P. indica* and *Rhizobium radiobacter* PCRE 10 and the values were statistically on par with those treated with single application of *Rhizobium radiobacter* PCRE 10 (12.66). On second month after transplanting, highest number of leaves (16.33) was observed in the treatment with combined application of *P. indica* and *Rhizobium radiobacter* PCRE 10 and the values were statistically on par with those treated with single application of *Rhizobium radiobacter* PCRE 10 (14.33). Observations taken in third, fourth and fifth months after transplanting, higher number of leaves were recorded in the treatment with combined application of *P. indica* and *Rhizobium radiobacter* PCRE 10, followed by single application of *P. indica.*

Observations taken in sixth and seventh month after transplanting, higher number of leaves was recorded in plants treated with *Rhizobium radiobacter* PCRE 10 alone (56 and 84 leaves respectively) and the values were statistically on par with those treated with combined application of *P. indica* and *Rhizobium radiobacter* PCRE 10.

A significantly increasing trend was observed in leaf area per plant of the plants treated with treated with combined application of *P. indica* and *Rhizobium radiobacter* PCRE 10. Maximum leaf area per plant was recorded in the treatment with combination of *Piriformospora indica* and *Rhizobium radiobacter* PCRE 10 from fourth month after transplanting and it was statistically on par with the treatment consisting of *Piriformospora indica* alone.

Observation on number of spikes harvested was taken eight months after transplanting. When the number of spikes harvested was analyzed statistically, there were no significant difference observed among the treatments. The highest value was recorded in the treatment with combination of *Piriformospora indica* and *Rhizobium radiobacter* PCRE 10.

Mean spike length of harvested berries was analyzed statistically, there was significant difference observed among the treatments

Observations in dry weight and fresh weight of berries were taken after harvesting the spikes in the 8^{th} month after transplanting. When the berry fresh weight as well as dry weight were analyzed statistically, there were no significant difference observed among the treatments. The highest value was recorded in the treatment involving single application of *P. indica*.

Results of the plant growth promotion experiment showed that there were significant differences in plant growth parameters between the effects of the combined application of *P. indica* and *Rhizobium radiobacter* PCRE 10, and all other treatments. Among all the growth parameter analysed, the maximum value was obtained in plants

treated with either combined or single application *P. indica* and *Rhizobium radiobacter* PCRE 10.

Roots of all the plants treated with *Piriformospora indica* and the combinations of *Piriformospora indica* with bacterial strain both as mixed inoculum were stained to assess the extent of root colonization by the fungal entophyte. Plants treated with *Piriformospora indica* alone showed highest root colonization with 35.5 percent followed by combination by *P. indica* with *Bacillus velezensis* PCSE 10 (31.11 percent), mixed inoculation of *Piriformospora indica* and *Rhizobium radiobacter* PCRE 10 (19.35 percent).

A pot culture experiment was carried out in already established bush pepper plants to study the effect endophytes and their combination in suppression of *P. capsici* induced foliar infection in the black pepper var. Panniyur- 1. Suppression of foliar infection by *P. capsici* in plants treated with the individual endophytes and their combination was studied by challenge inoculation with the pathogen on the foliage. Following artificial inoculation with the pathogen, the lowest lesion size was observed in plants treated with combination of *P. indica* and *Rhizobium radiobacter* PCRE10 (0.43 cm) which caused 10.41% disease suppression over the pathogen control with the lowest disease index of 0.2. No symptoms of disease were observed in chemical check and absolute control.

Results of the current investigation suggest that mixed inoculation with *P. indica* and *Rhizobium radiobacter* PCRE 10 which are compatible each other *in vitro* studies is more efficient than single inoculation of the biological agents for improving growth in bush pepper.

Endophytic bacteria used in this study were found to have biocontrol properties against foot rot pathogen, *P. capsici*. Apart from the biocontrol property, many of the bioagents had the potential to promote the growth of bush pepper plants. Biorational approaches involving bacterial endophytes, root endophytic fungus *P. indica* and their

combinations may have positive influence on the reduction of incidence of foliar infection of foot rot pathogen in controlled experimental conditions.

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Appendices

APPENDIX- 1

COMPOSITION OF MEDIA USED

1. Nutrient agar

Peptone	-	5 g
NaCl	-	5g
Beef extract	-	3g
Agar	-	20g
Distilled water	-	1000 ml

Peptone, NaCl and beef extract were dissolved in 500 ml distilled water and volume made up to 1000 ml. 20 g agar- agar was added into this mixture and autoclaved at 15 lbs pressure and 121 °C for 15 min.

2. Potato Dextrose Agar

Peeled and sliced potato	-	200 g
Dextrose	-	20 g
Agar-agar	-	20 g
Distilled water	-	1000 mL

Potatoes were boiled in 500 mL of distilled water and the extracts was collected by filtering through a muslin cloth. Agar-agar was dissolved separately in 500 mL of distilled water. The potato extract was mixed in the molten agar and 20 g of dextrose was dissolved in the mixture. The volume was made upto 1000 ml with distilled water and medium was sterilized at 15 lbs pressure and 121°C for 15 minutes.

APPENDIX- 2

COMPOSITION OF STAINS USED

1. Lactophenol preparation

Lactic acid	-	10 ml
Phenol	-	10 ml
Glycerol	-	20 ml
Water	-	20 ml

Lactic acid, Phenol, Glycerol and Water are mixed together and kept in glass jar.

2. Lactophenol-tryphan blue

Mix 50 mg tryphan blue in 100 ml lactophenol blue

KERALA AGRICULTURAL UNIVERSITY

COLLEGE OF AGRICULTURE, VELLAYANI

PLANT GROWTH PROMOTION AND FOOT ROT DISEASE SUPPRESSION IN BLACK PEPPER USING FUNGAL AND BACTERIAL ENDOPHYTES

by

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ABSTRACT

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ABSTRACT

The study entitled “Plant growth promotion and foot rot disease suppression in black pepper using fungal and bacterial endophytes” was conducted in the Department of Agricultural Microbiology, College of Agriculture, Vellayani during the period 2018 – 2020. The objective of the study was to assess and evaluate the compatibility of the root endophytic fungus *Piriformospora indica* and two endophytic bacterial strains *Bacillus velezensis* PCSE 10 and *Rhizobium radiobacter* PCRE 10 and their effect on growth promotion in bush pepper and foot rot disease suppression in black pepper.

Experiments comprised both *in vitro* and *in vivo* studies. *In vitro* interaction of bacterial bioagents and *P. indica* using dual culture plate assay was done to assess the compatibility between them. In PDA plates *B. velezensis* PCSE10 inhibited the growth of *P. indica* with an inhibition zone of 4 mm on 6^{th} day after bacterial inoculation.

Virulent isolate of foot rot pathogen *Phytophthora capsici* was isolated from infected black pepper leaves on PDA medium. The pathogenicity of the strain was proved by artificial inoculation on black pepper leaves.

In vitro antagonism shown by the bacterial bioagents against the pathogen was evaluated using dual culture plate assay. All the three bioagents were found to have *in vitro* antagonism against *P. capsici.* In the dual culture plate assay zone of inhibition was produced by *B. velezensis* PCSE 10 (4.67 mm) and *R. radiobacter* PCRE 10 (1.03 mm).

The dual culture plate assay was followed by a detached leaf assay using the two endophytic bacterial isolates. There was significant difference in lesion size on control leaves sprayed with sterile water and leaves sprayed with the selected isolates. The minimum lesion size was observed in leaves treated with *R. radiobacter* PCRE10 (1.43 cm) which caused 52.33 % disease suppression over control which was on par with leaves treated with *B. velezensis* PCSE 10 with lesion size of 2.18 cm and 27.33% disease suppression over control. The maximum lesion size was observed in control leaves sprayed with distilled water (3.0 cm).

Both the isolates produced IAA under *in vitro* conditions. *R. radiibacter* PCRE 10 was found to produce 4.62 µg/ml and 8.79 µg/ ml IAA without and with L- Trypthophan respectively. *B. velezensis* PCSE 10 produced 3.77 µg/ml and 4.92 µg/ml IAA in absence and presence of L- Tryptophan respectively. *R. radiobacter* PCRE 10 was also positive for ammonia production.

A pot culture experiment was conducted to study the effect of the different treatments on growth promotion and foot rot suppression in bush pepper. The experiment was laid out in CRD with six treatments and three replications and observations were taken at 30 days interval. The treatments comprised fungal and bacterial endophytes individually along with combinations of both fungal and bacterial endophytes and an uninoculated control. Bioagents were applied during the production of rooted cuttings and plants transplanted to pots filled with unsterile garden soil.

The plant growth promotion aspects of the endophytes were studied by analysing the biometric characters of bush pepper var. Panniyur -1 at monthly intervals. The results revealed that the maximum leaf number was observed in plants treated with combination of *P. indica* and *R. radiobacter* PCRE10. Maximum leaf area per plant was recorded in the treatment with combination of *P. indica* and *R. radiobacter* PCRE 10 at fourth month after transplanting.

Single application of *P. indica* resulted in the highest spike length. The highest number of spikes harvested per plant was recorded in the treatment with combination of *P. indica* and *R. radiobacter* PCRE 10. When the berry fresh weight and dry weight were analyzed statistically, there was no significant difference observed among the treatments. The highest value was recorded in the treatment involving single application of *P. indica*. Plants treated with *P. indica* alone showed highest root colonization of 35.50 percent followed by combination by *P. indica* and *B. velezensis* (31.11 per cent) and *P. indica* and *R. radiobacter* PCRE10 (19.35 percent).

Suppression of foliar infection by *P. capsici* in plants treated with the individual endophytes and their combination was studied by challenge inoculation with the pathogen on the foliage. Following artificial inoculation with the pathogen, the lowest lesion size was observed in plants treated with combination of *P. indica* and *R. radiobacter* PCRE10 (0.44 cm) which caused 33.33% disease suppression over the pathogen control with the lowest disease index of 0.2.

The *in vivo* study for plant growth promotion revealed that plants treated with combination of *P. indica* along with *R. radiobacter* PCRE10 and single inoculation of *P. indica* performed better than all other treatments.

സംഗ്രഹം

വെള്ളായണി കാർഷിക കോളേജിലെ കാർഷിക മൈക്രോബയോളജി വിഭാഗത്തിൽ 2018 - 2020 കാലയളവിൽ "കുമിൾ, ബാക്ടീരിയൽ എൻഡോഫൈറ്റുകൾ ഉപയോഗിച്ച് കുരുമുളക് ചെടികളുടെ വളർച്ച പ്രോത്സാഹനം, കാൽ ചെംചീയൽ രോഗം അടിച്ചമർത്തൽ" എന്ന തലക്കെട്ടിലുള്ള പഠനം നടത്തി. റൂട്ട് എൻഡോഫൈറ്റിക് കുമിളായ *പിരിഫോർമോസ്പോറ ഇൻഡിക്കയും*, *ബാസിലസ് വെലെസെൻസിസ്* പിസിഎസ്ഇ 10, *റൈസോബിയം റേഡിയോബാക്ടർ* പിസിആർഇ 10 എന്നി രണ്ട് എൻഡോഫൈറ്റിക് ബാക്ടീരിയകളും തന്നിലുള്ള അനുയോജ്യതയും കുറ്റി കുരുമുളകിലെ വളർച്ചയെ പ്രോത്സാഹിപ്പിക്കുന്നതിലും കാൽ ചെംചീയൽ രോഗത്തെ അടിച്ചമർത്തുന്നതിലും ഉള്ള കഴിവ് വിലയിരുത്തുകയും ചെയ്യുകയായിരുന്നു പഠനത്തിന്റെ ലക്ഷ്യം.

ബാക്ടീരിയൽ ബയോജെന്റുകളുടെയും *പിരിഫോർമോസ്പോറ ഇൻഡിക്കയും* തമ്മിലുള്ള അനുയോജ്യത വിലയിരുത്തുന്നതിനായി ഡ്യുവൽ കൾച്ചർ പ്ലേറ്റ് അസ്സെ ഉപയോഗിച്ചു. പിഡിഎ പ്ലേറ്റുകളിൽ *ബാസിലസ് വെലെസെൻസിസ്* പിസിഎസ്ഇ 10, *പിരിഫോർമോസ്പോറ ഇൻഡിക്ക*യുടെ വളർച്ചയെ തടഞ്ഞു. കാൽ ചെംചീയൽ രോഗകാരിയായ കുമിൾ ബാധിച്ച കുരുമുളക് ഇലകളിൽ നിന്നും *ഫൈറ്റോപ്തോറ കാപ്സിസി*, പിഡിഎ എന്ന മാധ്യമം ഉപയോഗിച്ച് വേർതിരിച്ചു. കുരുമുളക് ഇലകളിൽ കൃത്രിമ കുത്തിവയ്പ്പിലൂടെ സമ്മർദ്ദത്തിന്റെ രോഗകാരിത്വം തെളിഞ്ഞു.

ഡ്യുവൽ കൾച്ചർ പ്ലേറ്റ് അസ്സെ ഉപയോഗിച്ച് രോഗകാരിക്കെതിരായ ബയോ ഏജന്റുകൾ കാണിച്ച ഇൻ വിട്രോ വൈരാഗ്യം വിലയിരുത്തി. മൂന്ന് ബയോ ഏജന്റുമാർക്കും *ഫൈറ്റോപ്തോറ കാപ്സി* ക്കെതിരെ ഇൻ വിട്രോ വൈരാഗ്യമുണ്ടെന്ന് കണ്ടെത്തി. ഡ്യുവൽ കൾച്ചർ പ്ലേറ്റ് അസ്സെയ്ക്ക് ശേഷം രണ്ട് എൻഡോഫൈറ്റിക് ബാക്ടീരിയകളും

ഉപയോഗിച്ച് വേർതിരിച്ച ഇലയി ൽ പരിശോധന നടത്തി. അണുവിമുക്തമായ വെള്ളത്തിൽ തളിക്കുന്ന നിയന്ത്രണ ഇലകളിലും ബാക്ടീരിയ തളിക്കുന്ന ഇലകളിലും നിഖേദ് വലുപ്പത്തിൽ കാര്യമായ വ്യത്യാസമുണ്ട്. *റൈസോബിയം റേഡിയോബാക്റ്റർ* പിസിആർഇ 10 (1.43 സെ.മീ) ഉപയോഗിച്ച് ചികിത്സിച്ച ഇലകളിലാണ് ഏറ്റവും കുറഞ്ഞ നിഖേദ് വലുപ്പം കണ്ടെത്തിയത്.രണ്ട് ബാക്ടീരിയകളും ഇൻ വിട്രോ സാഹചര്യങ്ങളിൽ ഇൻഡോൾ അസെറ്റിക് ആസിഡ് ഉൽപാദിപ്പിച്ചു. *റൈസോബിയം റേഡിബാക്റ്റർ* പിസിആർഇ 10 യഥാക്രമം മൈക്രോഗ്രാം/ മില്ലി, 4.62 മൈക്രോഗ്രാം/ മില്ലി എൽ-ട്രിപ്റ്റോഫാൻ സാന്നിധ്യത്തിലും അസാന്നിധ്യത്തിലും ഉൽപാദിപ്പിക്കുന്നതായി കണ്ടെത്തി. റൈസോബിയം *റേഡിയോബാക്റ്റർ* പിസിആർഇ 10 അമോണിയ ഇൻ വിട്രോ ഉൽപാദിപ്പിച്ചു.

കുറ്റി കുരുമുളകിന്റെ വളർച്ച പ്രോത്സാഹിപ്പിക്കുന്നതിലും കാൽ ചെംചീയൽ അടിച്ചമർത്തുന്നതിലുമുള്ള വ്യത്യസ്ത ചികിത്സകളുടെ ഫലത്തെക്കുറിച്ച് പഠിക്കാൻ ഒരു പോട്ട് കൾച്ചർ പരീക്ഷണം നടത്തി. ആറ് ചികിത്സകളോടെ പൂർണ്ണമായും ക്രമരഹിതമായ ഡിസൈനിൽ പരീക്ഷണം നടത്തി. കുമീൾ, ബാക്ടീരിയ എൻഡോഫൈറ്റുകൾ എന്നിവയുടെ ഒറ്റയായ പ്രയോഗവും, കൂടാതെ അവയുടെ സംയോജനവും, നിയന്ത്രണാതീതമായ നിയന്ത്രണവും ചികിത്സയിൽ ഉൾപ്പെടുന്നു. വേരുറപ്പിക്കാനായി തണ്ടുകൾ കൂടയിൽ നടുന്ന സമയത്തും വേരുറപ്പിച്ച ചെടികൾ അസ്ഥിരമായ തോട്ടം മണ്ണ് നിറച്ച ചട്ടിയിലേക്ക് പറിച്ചു നടുന്ന സമയത്തും എന്നിവയുടെ ഉൽപാദനത്തിൽ ബയോ ഏജന്റുകൾ പ്രയോഗിച്ചു.

കുറ്റി കുരുമുളക് ഇനം പന്നിയൂർ 1 ന്റെ ബയോമെട്രിക് പ്രതീകങ്ങൾ വിശകലനം ചെയ്തുകൊണ്ട് എൻഡോഫൈറ്റുകളുടെ സസ്യവളർച്ച പ്രോത്സാഹന വശങ്ങൾ പ്രതിമാസ ഇടവേളകളിൽ പഠിച്ചു. *പിരിഫോർമോസ്പോറ ഇൻഡിക്ക*, *റൈസോബിയം റേഡിയോബാക്റ്റർ*

പിസിആർഇ 10 എന്നിവയുടെ സംയോജനത്തിൽ ചികിത്സിച്ച സസ്യങ്ങളിൽ പരമാവധി ഇല വിസ്തീർണ്ണം ഇലകളുടെ എണ്ണം കണ്ടെത്തിയതായി ഫലങ്ങൾ വെളിപ്പെടുത്തി. പിരിഫോർമോസ്പോറ *ഇൻഡിക്ക*യുടെ ഒറ്റയ്ക്കുള്ള ആപ്ലിക്കേഷൻ ഏറ്റവും ഉയർന്ന തിരി ദൈർഘ്യത്തിന് കാരണമായി. *പിരിഫോർമോസ്പോറ ഇൻഡിക്ക*യിൽ മാത്രം ചികിത്സിച്ച സസ്യങ്ങൾ ഏറ്റവും ഉയർന്ന റൂട്ട് കോളനിവൽക്കരണം 35.50 ശതമാനം രേഖപ്പെടുത്തി.

വ്യക്തിഗത എൻഡോഫൈറ്റുകളും അവയുടെ സംയോജനവും ചികിത്സിക്കുന്ന സസ്യങ്ങളിൽ *ഫൈറ്റോപ്തോറ കാപ്സിസി* ഫോളിയർ അണുബാധയെ അടിച്ചമർത്താനുള്ള ശേഷിയെ പറ്റി പഠനം നടത്തുകയും ചെയ്തു. രോഗകാരിയായ കുമിളിൻറെ കൃത്രിമ കുത്തിവയ്പ്പിനെത്തുടർന്ന്, *പിരിഫോർമോസ്പോറ ഇൻഡിക്ക, റൈസോബിയം റേഡിയോബാക്ടർ* പിസിആർഇ 10 (0.44 സെ. മീ) എന്നിവയുടെ സംയോജനത്തിൽ ചികിത്സിച്ച സസ്യങ്ങളിൽ ഏറ്റവും കുറഞ്ഞ നിഖേദ് വലുപ്പം കണ്ടെത്തി, ഇത് രോഗകാരി നിയന്ത്രണത്തെക്കാൾ 33.33% രോഗം അടിച്ചമർത്താൻ കാരണമായി.

റൈസോബിയം റേഡിയോബാക്ടർ പിസിആർഇ 10, *പിരിഫോർമോസ്പോറ ഇൻഡിക്ക* എന്നിവ സംയോജിപ്പിച്ച് ചികിത്സിച്ച കുറ്റി കുരുമുളകിന്റെ വളർച്ചയും കാൽ ചെംചീയൽ രോഗകാരിയായ കുമിളുകൾ അടിച്ചമർത്തുന്നതിലുമുള്ള തിരഞ്ഞെടുത്തു.