EVALUATION OF BIOFILM BASED MICROBIAL ANTAGONISTS FOR THE MANAGEMENT OF SOIL BORNE DISEASES AND GROWTH PROMOTION IN COWPEA (*Vigna unguiculata* L. Walp)

By

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(2015-11-121)

THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Evaluation of biofilm based microbial antagonists for the management of soil borne diseases and growth promotion in cowpea (*Vigna unguiculata* L. Walp)"** 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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ABBREVATIONS

µg - Microgram

ml - Milliliter

cfu - Colony forming unit

h - Hour

g - Gram

L - Litre

mm - Millimeter

PDA - Potato dextrose agar

NA - Nutrient agar

CAS - Chrome Azurol S

HCN - Hydrogen cyanide

IAA - Indole acetic acid

C - Carbon

N - Nitrogen

P - Phosphorus

K - Potassium

EPS - Exopolysaccharides

TCP - Tissue culture plate

TM - Tube method

CRA - Congo red agar

NaCl - Sodium chloride

TSB - Trypticase soy broth

OD - Optical density

ELISA - Enzyme linked immune sorbent assay

rpm - Rotation per minute

°C - Degree Celsius

Kg - Kilo gram

MAI - Months after inoculation

CRD - Completely randomized design

KAU - Kerala Agricultural University

Ha - Hectare

t - Ton

CEC - Cation exchange capacity

EC - Electrical conductivity

OC - Organic carbon

DAI - Days after inoculation

DAS - Days after sowing

1. INTRODUCTION

Indian agriculture is highly dependent on the use of chemical fertilizers, growth regulators and pesticides to obtain maximum yield for the ever growing population. With increasing use of chemicals, problems such as environmental pollution, health hazards, interruption of ecological nutrient cycle and destruction of biological communities are associated with agriculture (Bedano *et al.*, 2006). Indiscriminate use of inorganic fertilizers and pesticides in the past have caused huge damage to our soil and agricultural ecosystems. As an alternative to the toxic inorganic chemicals, plant beneficial microorganisms have been exploited based on their nutrient acquisition ability, plant growth promotion and biological control of pests and diseases. These microbial inoculants play an important role as eco-friendly, non-bulky and low cost agricultural inputs. *Trichoderma* sp. and *Bacillus* sp. are the most successful biocontrol agents, which are commercially exploited in India (Dawar, *et al.*, 2010: Pandya, *et al.*, 2011). *Trichoderma* sp. can parasitize the fungal pathogens by several mechanisms. It can induce systemic and localized resistance to plants against many plant pathogens. *Trichoderma* sp. are also involved in promoting plant growth by stimulating many enzymes and pathogenesis related protein in plants. *Bacillus* sp. is the most consistent plant growth promoter. It protects the plants from pathogenic microorganisms through various mechanisms (Cherif *et al.*, 2016). Some strains of *Bacillus* sp. enhance plant growth by releasing phytohormones (IAA), acids (HCN) and have the ability to solubilise P from soil reserves and improve the P uptake in plants.

Inconsistent field performance often restricts the use of many biocontrol agents and plant growth promoting rhizobacteria (PGPR). Both biotic and abiotic factors could affect bio agents under laboratory and field conditions. Inconsistent field performance could be attributed to adaptability to a non-native soil, negative effects of interaction with existing microbes in crop environment and incompatibility in colonizing different crop plants (Elsas *et al.*, 1986). Introduction of new microbe into the plant ecosystem might have definite impact on the native microbial balance in the soil and crop environment. All these reasons affect the survivability of the inoculated

biocontrol agents. Failure of biocontrol agents to survive in soil results in the development of plant diseases, causing huge crop loss. Improvement in the survivability of biocontrol agents have become a major area of concern.

With the advances in knowledge regarding various traits of microorganism, there is a need to improve the survivability of the bioinoculants in the soil to eliminate the inconsistent performance of the biocontrol agents. In this context, biofilm based bioinoculants is an important novel approach which has the ability to protect the bioinoculants from various environmental stress factors such as UV radiation, extreme pH, osmotic shock, dehydration, antimicrobial substances and predators (Romanova *et al.*, 2006). A biofilm comprises microbial cells and sticky extracellular polymeric substance (EPS) which provide structure and protection, and are commonly found in natural environment. Biofilm based bioinoculants are known to take part in soil ferility management, nutrient uptake, higher rate of biological nitrogen fixation, release of organic acids, phosphate solubilisation and helps in successful management of plant diseases (Jayasinghearachich and Seneviratne, 2004)

Cowpea is one of the important legume crops which is ranked among the top-five legume crops in the world. Major characteristics like good protein quality, high nutritional value, both drought and heat tolerant, higher biological nitrogen fixing ability has made this crop to grow worldwide (Hall *et al.*, 2003). However, pests and diseases hampers crop establishment, impairs seed quality and reduces the yield. Fungal diseases like collar-rot (*Rhizoctonia solani*), root rot (*Pythium aphanidermatum*), anthracnose (*Colletotrichum lindemuthianum)*, powdery mildew (*Erysipheae polygoni*) and other soil borne diseases have become a major concern in cowpea (Satish *et al.*, 2000). There is a need to increase the emphasis on use of eco-friendly approach, such as bioinoculants, to provide a long lasting solution. Bioinculants applied to soil, shows good results but survivability in the soil for a long period is affected because of many biotic and abiotic factors. The studies on biofilm based microbial antagonists for the management of cowpea diseases have not been carried out in Kerala.

Hence, the present study was under taken on "Evaluation of biofilm based microbial antagonists for the management of soil borne diseases and growth promotion in cowpea (*Vigna unguiculata* L. Walp)" with the following objectives:

- To increase the survivability of *Trichoderma* sp. and *Bacillus* sp. through biofilm production
- To evaluate the biofilm based biocontrol agents for the management of *Rhizoctonia solani* and *Pythium aphanidermatum* and also growth promotion in cowpea.

2. REVIEW OF LITERATURE

One of the major constraints in the bioinoculant application is the survivability of the microorganisms under field conditions. Even though, the inoculants are applied to the soil, both biotic and abiotic stresses affect its survivability. Hence, there is a need to explore the various methods to increase the survivability in soil. Cowpea is one of the important vegetable crops of Kerala, which is affected by several soil borne diseases. The literature related to diseases and biofilm based inoculants of cowpea are reviewed under this chapter.

2.1. COWPEA AND ITS IMPORTANCE

Cowpea (*Vigna unguiculata* L. Walp) is one of the most popular nutritious legume crop, traditionally grown in humid tropics of Kerala. Cowpea is believed to have been originated from western Africa and later spread to Asia. In India, it is grown as a sole crop, mixed crop, inter crop combinations and in 2015-16, it was cultivated on about 1241 ha. in the country. It is an inexpensive source of vegetable protein and vitamins, which must be included in our daily diet. The seed protein ranged from 23 to 57 per cent, in which amino acids like tryptophan and lysine were abundant (Tarawali *et al.*, 2003). It also contains lesser quantity of fat and high amount of fibre, which prevent heart disease by lowering the lipoprotein (Philip *et al.*, 2003). Due to favourable agro-climatic conditions, the crop has gained much importance in Kerala.

2.2. Diseases of cowpea

Major constraints hindering the cowpea production are the occurrence of different types of diseases. Though, cowpea is gaining importance among the farmers, it is severly infected by many fungal, bacterial and viral diseases.

Kossou *et al.* (2011) reported that pests, diseases and weeds like aphids, *Sclerotium rolfsii* and *Ipomea* could be a major constraints in cowpea production. Low soil fertility, poor farming practices, lack of labours and biological constraints also contributes in limiting cowpea production (Abadassi, 2014).

2.2.1. Collar rot

Collar rot disease is one of the major disease in cowpea during seedling stage. It is caused by *Rhizoctonia solani* and was the most severe soil borne disease reported in Kerala. Collar rot is characterized by oval or spindle shaped black to brown lesions at soil level near collar region, girdling and basal portion of the stem. Leaves turns yellow and finally plant wilts (Vavilapalli *et al*., 2014).

Singh *et al.* (2014) reported that collar rot disease of cowpea caused by *R. solani* was successfully controlled by the application of mushroom spent compost based *T. harzianum* and observed reduction in the seedling mortality of cowpea.

Chagas *et al.* (2015) studied the combined application of *Trichoderma* and *Rhizobium* on cowpea growth promotion and management of *R. solani.* The study revealed that combined application at 15 DAP had highest number of plant and survival against *R. solani.*

2.2.2. Anthracnose

Anthracnose is a severe disease in vegetable cowpea caused by *Colletotrichum lindimuthianum.* It has been ranked as the fourth most studied phyto-pathogenic fungi, being surpassed by *Fusarium, Phytophtora* and *Rhizoctonia* sp. (Hyde *et al.*, 2009).

In India, first report of anthracnose disease incidence was reported from Maharastra in 1966 (Rao, 1966). *Colletotrichum* sp. uses wide range of mechanisms for invading host plant tissue like intracellular hemibiotrophs to subcuticular intramural necrotrophs for infection (Perfect *et al.*, 2002).

The fungal pathogen is known to cause 35 to 50 % crop loss and could be complete crop loss during seedling stage (Varma and Langurk, 1988). *Colletotrichum* sp. produces fruiting body acervuli, which is a pale brown in color, hemi spherical and composed of conidiophores and conidia (Watanabe, 2010).

In response to pathogen infection, plant produces low molecular weight antimicrobial compounds such as phytoalexins and antipeptide small proteins. These compounds inhibit the growth and proliferation of pathogen by inhibiting the

synthesis of fungal cell wall / disrupting the cell wall resulting in cell lysis (Selitrennikoff, 2001).

Mohammed (2013) reported that seed dressing with *T. harzianum* and *T. viride* before sowing could restrict the *Colletotrichum* infection upto 89.44 %, which was better than the synthetic fungicide (vitavax- 200) in common bean.

2.2.3. Powdery mildew

Powdery mildew of cowpea caused by *Erysipheae polygoni* is one of the major disease that might reduce the yield upto 30 per cent. Powder mildew fungi act as biotrophic parasites invading only epidermal cells (Huckelhoven, 2005: Boesewinkel, 1980).

Powdery mildew on cowpea produces white mycelial cell mass on all parts of the plants including pods, leaves and stem. Disease symptoms starts initially from older leaves and extends towards tip portion as the disease progresses. Under severe disease infection, white mycelial growth covers the entire plant and later plant tissue turns into black (Mishra *et al.*, 2005).

2.2.4. Cowpea Mosaic virus

Cowpea mosaic virus (CPMV) is a deadly viral disease transmitted by aphids. The virus has thermal inactivation point of about 60-62 °C and has survivability under *in vitro* for 5 days. (Lovisolo and Conti, 1996).

Carette *et al.* (2000) reported that replication of CPMV after infection inside the host plant induces the formation of membranous vesicles. It was also reported that, CPMV induced extensive proliferation of endoplasmic reticulum, which is indirectly associated with the formation of membranous vesicles.

2.3. Biological control of cowpea diseases

Plant diseases may cause around 25-30 per cent crop losses, which impairs the quality of the produce and the economic value of the crop. Biological control has

become a potential and promising strategy in management of many soil borne and foliar diseases, which is both eco-friendly and cost effective.

Pseudomonas fluorescens showed maximum antagonistic activity than *Bacillus subtilis* in biological control of cowpea anthracnose caused by *Colletotricum destructivum* (Akinbode and Ikotun, 2008).

Afouda *et al.* (2012) concluded that seed treatment of cowpea with *Bacillus subtilis* along with cultural practices reduced the *Macrophomina phaseolina* population and it lowered the incidence of pathogen by 82.29 % over control under *in vitro*. Almoneafy *et al.* (2012) reported that *Bacillus* isolates showed significant differences in suppression of bacterial wilt of tomato caused by *Ralstonia solanacearum* through siderophore production and promoted plant growth by the secretion of indole acetic acid.

Sivasakti *et al.* (2013) studied the production of plant growth promoting substances released by *Bacillus subtilis* and *Pseudomonas fluorescens* which enhanced the plant growth.

Antagonists like *Bacillus* and *Trichoderma,* when used as seed or soil treatment at the time of planting could decrease the disease incidence of cowpea soil borne pathogens, increased the plant survival and restored the biological N_2 fixation (Ghoniem and Belal, 2013).

Use of microbial antagonists like *Trichoderma* sp., *Pseudomonas fluoroscens* and *Bacillus subtilis* were effective against plant pathogenic fungi. Combining these biocontrol agents with other plant growth promoting microorganisms like *Rhizobium* sp., *Glomus* sp. and *Bacillus megaterium* could yield better results in growth promotion of plants, nutrient management and suppression of diseases (Naik *et al.* 2014).

2.3.1. *Bacillus* as a biocontrol agent for cowpea disease managemnet

Bacillus sp. is a Gram positive, aerobic bacteria with biocontrol activity including the production of diverse antibiotics, formation of viable spores, promotion of plant growth and a ubiquitous presence in soil (Bais *et al.*, 2004).

Dawar *et al.* (2010) reported that *Bacillus subtilis* recorded maximum inhibition zone against *Macrophomina phaseolina* and *Rhizoctonia solani* under *in vitro* and reported significant increase in shoot length, shoot weight, root length and root weight in cowpea, when *Bacillus* sp. was used as seed dressing.

Singh *et al.* (2012) reported that combination of *Bacillus firmus* as seed coating and *Aspergillus versicolor* as soil applicant resulted in improved growth promotion and greater reduction in cowpea charcoal rot disease under arid soil condition.

Sunar *et al.* (2015) reported that *Bacillus altitudinis* (BRHS/S-73) isolated from Darjeeling (India) was capable of enhancing the defense related key enzymes such as phenylalanine ammonia lyase, peroxidase, β-1,3-glucanase, and chitinase in both roots and leaves and was efficient in suppression of root rot disease of *Vigna radiata* caused by *Thanatephorus cucumeris.*

2.3.1.1. Mechanism of action by Bacillus

2.3.1.1.1. Siderophore production

Bacterial siderophores are the low molecular weight iron chelating small peptide molecules having high Fe^{3+} chelating affinity and responsible for Fe^{3+} solubilisation and transport into bacterial cell.

Iron (Fe^{3+}) is reduced to Fe^{2+} on the membrane of both Gram positive and Gram negative bacteria via, a gating mechanism linking the inner and outer membranes. During this reduction process, the siderophore may be destroyed/recycled and binding of the siderophore to metal increases the soluble metal concentration (Rajkumar *et al.*, 2010).

Siderophores produced by *Bacillus subtilis* CTS-G24 drastically reduced the availability of ferric ions in the soil and inhibited the growth of the pathogenic microflora in the soil (Patil *et al.*, 2014).

In a study conducted by Santos *et al.* (2014), the results indicated that carbon source, aeration and glycerol affects the siderophore production in *Bacillus megaterium* and siderophore production was not related to sporulation of the bacterium.

2.3.1.1.2. Hydrogen cyanide production

Hydrogen cyanide (HCN) is a secondary metabolite produced by many antagonistic microorganisms from glycine. It inhibits the electron transport system, thereby disrupting the energy supply to the cell leading to the death of the organism. It inhibits the proper functioning of enzymes and natural receptors by reversible mechanism of inhibition (Corbett, 1974).

Reetha *et al.* (2014) reported that HCN producing *Bacillus* sp. was efficient in inhibiting the growth (69.2 %) of *M. phaseolina* under *in vitro*.

Cherif *et al.* (2016) reported that HCN produced by the *Bacillus* sp. were involved in the suppression of pathogens like *Pythium ultimum* in cucumber and *Fusarium oxysporum* in tomato and also reported that gene *hcn* was responsible for the release of this metabolite.

2.3.1.1.3. Indole acetic acid production

Indole-3-acetic acid (IAA) is an essential plant hormone and a best-characterised auxin, which is essential for the growth and development of the plants. Tryptophan is believed to be the precursor of IAA. It promotes plant growth by rapid cell division, elongation and cell differentiation (Srivastava and Kumar, 2011).

Datta *et al.* (1982) concluded that *Bacillus firmus* enhanced the rice grain yield by the production of indole-3-acetic acid and by phosphorus solubilisation in rice.

Several IAA biosynthetic pathways have been identified although; the best-characterized biosynthetic pathways for conversion of *Trp* to IAA was indole-3-acetamide pathway and indole-3-pyruvate pathway (Patten and Glick, 1996).

Deepa *et al.* (2010) reported that *Bacillus* sp. producing IAA influenced the growth and development of plant roots, thereby enhancing the nutrient uptake and contributed in increasing the P availability in the rhizosphere.

2.3.2. *Trichoderma* as biocontrol agent for cowpea

Trichoderma sp. is a soil inhabiting, filamentous fungi which belongs to the order *Hypocrea. Trichoderma* sp. is the most common biocontrol agents commercially exploited to control many phytopathogenic fungi (Schubert *et al.*, 2008). It has become a major biocontrol agent to manage several crop diseases by parasitizing the pathogen and antibiotics production (Pandya *et al.*, 2011).

Pan and Das (2011) stated that seed priming with *T. harzianum* and vermicompost based *T. harzianum* with 20 % neem cake showed significant reduction in the root and collar rot of cowpea caused by *Rhizoctonia solani.* It also promoted seedling vigour, germination, girth and increased the number of branches and pods in cowpea.

Trichoderma sp. plays an important role as biocontrol agent by their multiple roles like biopesticide, bioherbicides and plant growth promotor (Sriram *et al.*, 2013).

Sain and Pandey (2016) reported that *T. harzianum* could inhibit the growth of *Pythium aphanidermatum* and *Fusarium oxysporium* upto 78.44 and 74.16 % respectively under *in vitro*. They also stated that seed treatment of okra and brinjal with *T. harzianum* resulted in increased plant height (brinjal 52.9 cm; okra 110.5 cm) and fruit yield (brinjal 328.3 g; okra 294.6 g).

2.3.2.1. Mechanism of action by Trichoderma

2.3.2.1.1. Mycoparasitism

Weindlind (1932) described the mycoparasitism in *Rhizoctonia solani* by the hyphae of *Trichoderma*, which coils around the hyphae, penetration and dissolution of host cytoplasm in pathogen.

Bankole and Adebanjo (2002) reported that *Colletotrichum truncatum*, which causes cowpea brown blotch disease, was parasitized by *Trichoderma viride* by coiling the hyphae and overgrowing on the pathogen.

It has been revealed that, *ech42* (a chitinolytic encoding gene) transcription was involved during mycoparasitic interaction of *Trichoderma harzianum* prior to physical contact with the host plant pathogen (Verma *et al.*, 2007).

2.3.2.1.2. Antibiosis

Increase in the antagonistic activity of *Trichoderma* sp. may be due to the production of various secondary metabolites which act as inhibitors to many phytopathogens. Khare *et al.* (2010) reported that mutant strain of *T. viride* (1433) showed significant antagonistic activity against *Pythium aphanidermatum* by the production of many volatile and non-volatile metabolites.

Patil *et al.* (2012) also showed that under *in vitro* condition, application of *Trichoderma* sp. reduced the *Pythium* growth and triggerd the plant mediated defence mechanism in respose to *Pythium* infection due to volatile and diffusible metabolites.

Pan *et al.* (2013) evaluated five different isolates of *Trichoderma* against *Rhizoctonia solani, Sclerotium rolfsii, Fusarium ciceri* and *Macrophomina phaseolina* under *in vitro* condtion and reported the successful inhibtion of mycelial growth of test pathogens by the production of both volatile and non-volatile inhibitors of *Trichoderma.*

2.3.2.1.3. Competition

Trichoderma sp. has superior character in utilization of nutrients and its mobilization in the soil compared to other microorganisms. It produced organic acids like gluconic, fumaric, citric acids which helped in solubilisation of insoluble phosphates, micronutrients in plants due to reduction in pH (Benitez *et al*., 2004).

Elad (2000) reported that *Trichoderma harzianum* was rhizosphere competent enough to extract nutrients in the soil and had capcity to suppress the growth of *Sclerotium rolfsii* in rhizosphere region.

2.3.2.1.5. Induced systemic resistance

In addition to other antagonistic mechanisms of *Trichoderma*, induction of resistance to host plants against pathogens have also been reported as indirect growth promotion factor (Zeilinger, 1999).

Some strains of *Trichoderma* can induce both systemic and localized resistance to several plant pathogens. Plants treated with *Trichoderma* in the root zone could produce more quantity of peroxidase, chitinase activity, deposition of callose-enriched wall appositions on the inner surface of cell walls and pathogenesis-related proteins (Howell, 2002).

Sharma *et al.* (2011) reported that the *Trichoderma* played a major role in management of plant diseases by the production of various kinds of enzymes with significant role in biocontrol activity like cell wall degradation, biotic and abiotic stress tolerance, hyphal growth, antagonism against most phytopathogenic microorganisms.

Howell (2003) reported that plants treated with *T. harzianum* recorded increased root area, root length, shoot length, leaf area and significant increase in the dry weight of the plant over the control.

2.4. Survivability of biocontrol agents in soil

Survival and functioning of commercial biofertilizers are inconsistent under field conditions due to heterogeneity of biotic and abiotic stress factors and competition with indigenous organisms (Nelson, 2004).

Soil temperature and moisture, pH, texture, oxygen and availibility of nutrients were the major limiting abiotic factors governing the survivability of the introduced biocontrol agents in the soil. Management of phytopathogens in the soil depends on the presence of supressive soils where many biocontrol agents *Trichoderma, Bacillus* and *Pseudomonas* have been detected. (Lowendorf *et al.*, 1981).

Popuation count of *Bacillus subtilis* (LU1241R) and *Bacillus polymyxa* (LU1133R) in non sterile soil conditions were very low and recovery per cent of applied bacteria from soil was not successful after one month of application (Bolstridge *et al.*, 2009).

Repeated freeze-thaw condition during winter caused *Bacillus subtilis* to show lesser motility and higher ionic strenghts caused greater survival. Survival strategies like biofilm formation and motility were found to be significantly influenced by these conditions (Asadishad *et al.*, 2014).

2.5. Biofilm based biocontrol agents

2.5.1. Biofilms

The term biofilm was coined by a marine microbiologist Claude ZoBell from La Scripps Institute of Oceanography while explaining the 'bottle effect' which means the levels of floating planktonic bacteria in sea water declines as they adhered to glass surfaces (Donlan, 2002).

Biofilm is a complex aggregation of microorganisms characterized by the excretion of a protective adhesive matrix. Cells living in a biofilm are embedded in this polymeric substances, which permits them to adhere and colonize the surface of different materials (Singh, 2006).

According to Costerton *et al.*, (1995), formation of biofilms in natural and industrial environments allows bacteria to develop resistance against bacteriophage, amoebae, chemically diverse biocides, host immune responses and antibiotics.

Bacteria can develop biofilms on a number of different surfaces, such as natural aquatic and soil environments, living tissues, medical devices or industrial or potable water piping systems (Donlan 2002; Flemming and Wingender, 2001). Attachment of bacteria to the substrate would increase the chances for adsorption of organic trace elements (Sheng *et al.*, 2008).

Biofilm formation by *B. subtilis* follows a distinct developmental pathway. During biofilm development, *B. subtilis* switches from flagellated, motile single cells to long chains of nonmotile cells that form parallel bundles (Branda *et al.*, 2005). This switching transition is under the negative regulation of SinR, a master regulatory repressor. Regulation is achieved by the repression of the *eps* operon which is responsible for the biosynthesis of the exopolyssacharide (Kearns *et al.*, 2005).

Sand and Gehrke (2006) found that cells with higher concentration of glucuronic acids and iron in the exopolymers increased the higher oxidation activity than those with lower concentration of these components. They also concluded that there was a correlation between the presence of iron in the the EPS and the bacterial metabolic rate.

Root surfaces are known to be continuously subjected to the two-way traffic of solutes from plants to soil and vice versa and microbial colonisation is generally regulated in a population density manner by quorum sensing (QS). This leads to expression of specific genes that allow the partners in a biofilm resembling a multicellular organism to communicate with each other, (Rudrappa *et al.*, 2008).

According to Rudrappa and Bais (2007), direct catechol treatment to *Bacillus subtilis* significantly reduced biofilm production by interfering in the transcription levels of the operons yqxM and epsA which are required for biofilm formation. They also showed that suppression of biofilm formation on the roots of *Arabidopsis thaliana* line *NahG* was regulated by the presence of catechol on the nahG root

surface, resulting in generation of reactive oxygen species (ROS) mediated down regulation of genes required for biofilm production in *Bacillus subtilis*.

2.6. Screening of biocontrol agents for biofilm formation

Biofilm formation is a developmental process in which bacteria undergo a regulated lifecycle change from a nomadic unicellular state to a sedentary multicellular state where subsequent growth results in structured communities and cellular differentiation.

Mathur *et al.* (2006) conducted an experiment to detect biofilm formation in 152 clinical isolates of *Staphylococcus* sp. using tissue culture plate (TCP), tube method (TM) and congo red agar (CRA) methods and concluded that TCP method was the most reproducible, accurate, and best method for screening biofilm with an advantage of quantitative estimation.

Deka (2014) reported that TCP method was the best and most reliable method for biofilm detection. Biofilm formation depends on temperature, pH and sucrose concentration in the medium. In this context, Sonkusale and Tale (2015) reported that 37 °C, pH of 7.5 and 10-15 % sucrose concentration were ideal for biofilm formation and also concluded that absorbance value in TCP method was considered as index of biofilm formation.

Kasim *et al.* (2016) screened 20 different bacterial isolates under varying NaCl concentration and reported that biofilm formation with increasing salt concentration. Results also revealed that bacterial inoculation were effective in managing deleterious salinity problem in barley when compared with control.

Soil microorganism and their interactions with the soil component are less understood. A study conducted by Ma *et al.* (2017) revealed that soil clay minerals like geothite, montmorillonite and kaolinite had significant effect on the *Bacillus subtilis* biofilm formation. They found that mineral induced cell lysis was the major key factor in inducing bacteria to form biofilm with the corresponding mineral.

2.7. Effect of Biofilm based inoculant on growth promotion and disease management

Seneviratne and Jayasinghearachchi (2003) reported that biofilm developed by nitrogen fixing bacteria and P solubilising fungi under *in vitro* conditions showed increased rate of biological nitrogen fixation and organic acid production, which directly influenced the synthesis of indole acetic acid like substances than monocultures.

Bais *et al.* (2004) revealed that wild type *B. subtilis* (strain 6051) produced stable, extensive anti-microbial biofilm in *Arabidopsis* and secretion of lipopeptide antibiotic, surfactin reduced the infection of *Pseudomonas syringe*. Jayasinghearachchi and Seneviratne (2004) reported that Bradyrhizobial-*Penicillium* sp. biofilm improved nitrogenase activity over bradyrhizobial isolate alone. Biofilmed inoculants also showed increased shoot growth, root growth, nodulation and N accumulation in soybean.

Biofilm inoculants are known to take part in soil fertility management. Application of *Bradyrhizobium japonicum* (SEMIA 5019)-*Penicillium* sp. biofilm increased N and P mineralization in the soil along with higher nitrogenase activity even under very high NO_3^- concentration. These biofilm inoculants enhanced the soil fertility status and helped in survival of rhizobia even in the absence of its host (Jayasinghearachich and Seneveratne, 2004).

The biofilmed inoculum could be used for the successful establishment of introduced beneficial microorganisms in plants for biological control of plant diseases. A *Pleurotus ostreatus* and *Pseudomonas fluorescens* based biofilm enhanced endophytic colonization of tomato by *Pseudomonas fluorescens*, compared to inoculation with *Pseudomonas fluorescens* alone under *in vitro* (Jayasinghearachich and Seneveratne, 2006).

Bacterial biofilm based biofertilizers with moderate dose of fertilizer application recorded increased root growth, higher soil carbon, more shoot-root ratio and significantly higher yield in tea plantations (Zavahir *et al.*, 2008).

Seneveratne *et al.* (2011) examined the effect of microbial biofilms of N_2 fixers in restoring deteriorated soils due to conventional farm practices in tea gardens. They reported that combined application of less chemical fertilizers along with biofilm based biofertilizers resulted in significant increase in biological nitrogen fixation and also contributed in increasing soil organic C by 20 % and reduced the leaf transpiration by 40 %.

Prasanna *et al.* (2011) studied the biofilms formed from *Anabaena torulosa* with *Azotobacter chroococcum*, *Pseudomonas striata*, *Serratia marcescens* and *Mesorhizobium ciceri* as co-cultures. They found higher rate of plant growth promoting activities with co-cultures than monocultures alone.

Swarnalaksmi *et al.* (2013) studied the performance of novel cyanobacterium *Anabaena torulosa* based bacterial (*Azotobacter*, *Mesorhizobium, Serratia and Pseudomonas*) biofilm in wheat. *Anabaena-Serratia* biofilm recorded highest acetylene reduction activity (40-50 %) even after 14 weeks of biofilm inoculation. *Anabaena –Pseudomonas* biofilm showed highest P uptake when compared with monoculture and dual culture of the inoculants.

According to Buddhika *et al.* (2014), there exists a controlled regulation of IAA production for increased plant growth. Fungal bacterial biofilms showed significantly increase in seed germination and vigour compared to monocultures.

Bidyarani *et al.* (2016) reported that the *Anabaena- Rhizobium* biofilmed inoculant recorded higher grain yield with increased nutrient uptake, soil microbial activity and soil available nitrogen in chickpea.

Das *et al.* (2016) developed *Trichoderma viride-Mesorhizobium ciceri* biofilm inoculants for the management of *Fusarium* wilt and growth promotion in chickpea. They reported that biofilm inoculant exhibited significant enhancement in seed germination (13-21 %) and recorded 10-11 % increase in the antifungal activity against *Fusarium oxysporum* f. sp. *ciceri.*

2.7.1. Effect of *Trichoderma* based *Bacillus* sp. biofilm on plant

Fungal-bacterial biofilms in the endophytic environment showed improved acidity and higher production of indole acetic acid like substances in the soil. These acids suppressed the growth of plant pathogens which was very important in the plant health and growth promotion. There was a negative relationship existed between the pH of the biofilm and IAA (Bandara *et al.*, 2006).

Triveni *et al.* (2012) developed a novel biofilm based biofertilizer using *Trichoderma viride* as fungal matrices and nitrogen-fixing and P-solubilizing bacteria as partners and used it as a biocontrol agent for different crops.

Prasanna *et al.* (2014) evaluated the efficiency of cyanobacterial formulations and biofilmed inoculants in legumes. They reported that use of biofilm based *Trichoderma viride* and *Bradyrhizobium* increased fresh and dry weight of plant by 20-45 % over microbial treatments. They also reported that *Anabaena-Trichoderma viride* biofilmed formulations recorded increased microbial activity and yield by 12-25 %.

Triveni *et al.* (2012) evaluated PGP traits of novel biofilms developed using *Trichoderma, Pseudomonas fluorescens* and *Bacillus subtilis* as partners. Such biofilms exhibited higher biochemical attributes like enhanced antifungal activity, ammonia, indole acetic acid (IAA) and siderophore production as compared to the monocultures and dual cultures.

Cotton seeds treated with *T. viride-B. subtilis* biofilm registered 20-25 % lower mortality rate, when compared to control and also reported to improve plant growth by better root colonisation and reduction in ashy stem disease severity in cotton (Triveni *et al.*, 2015).

The review indicates that the biofilm based inoculants have the potential to tolerate biotic and abiotic factors and could be a novel approach to increase the survivabiliy of inoculants in soil. The literature search has also indicated that no studies have been carried out on the management of major soil borne diseases of cowpea in Kerala using biofilm based inoculants.

3. MATERIALS AND METHODS

The present studies on, "Evaluation of biofilm based microbial antagonists for the management of soil borne diseases and growth promotion in cowpea (*Vigna unguiculata* L. Walp)" was carried out at the Department of Agricultural Microbiology, College of Horticulture, Vellanikkara, during 2015-17. Details of materials used and the methods followed are presented below.

3.2.1. Collection of rhizosphere soil from cowpea growing areas

A survey was conducted on cowpea growing areas of Thrissur district and rhizosphere soil samples were collected from ten different locations. Six rhizosphere soils from healthy cowpea plants and four samples from collar rot infected plants were collected. The rhizosphere soil samples were subjected to quartering technique and the samples were stored in refrigerator for further use.

3.2.2. Isolation and enumeration of *Trichoderma* sp. and *Bacillus* sp. from the rhizosphere soil

Trichoderma and *Bacillus* were isolated and enumerated from rhizosphere soils by serial dilution plate technique (Chen *et al.*, 2016).

Ten gram of each soil sample was weighed and mixed with 90 ml sterile water to get 10^{-1} dilution. After shaking for 15 minutes one millilitre of suspension from 10^{-1} dilution was transferred to another test tube containing 9 ml sterile water to get 10^{-2} dilution. It was repeated until 10^{-6} dilution were obtained. One millilitre of suspension from 10^{-4}, 10^{-5}, 10^{-6} dilutions were transferred to sterile Petri dish separately and molten and cooled culture media (*Trichoderma* selective media for *Trichoderma* and Nutrient agar medium for *Bacillus*) were poured. The Petri dishes were rotated clockwise and anticlockwise direction to facilitate uniform mixing of the suspension. After solidification, the plates were kept for 2-3 days in the case of *Bacillus* and 5-7 days in the case of *Trichoderma*. After incubation, the number of colonies formed were counted and expressed as colony forming units (cfu) per gram of soil.

3.3.1. Purification and maintenance of predominant isolates

Nine predominant isolates of *Trichoderma* and five *Bacillus* isolates were obtained and purified using standard protocols.

3.3.2. Morphological and cultural characterization of *Trichoderma* isolates

Cultural and morphological characters of nine *Trichoderma* isolates were studied (Shah *et al*., 2012). Observations on shapes and colour of conidia, the branching patterns of conidiophores were observed under microscope. Cultural characters like number of concentric rings, colony form, and colony colour were also studied on PDA

3.3.3. Morphological, cultural characterization of *Bacillus* isolates

3.3.3.1. Morphological characterization of Bacillus isolates

For morphological studies, 24 h old *Bacillus* sp. isolates were used. Gram reaction, endospore and shape of the *Bacillus* were observed under oil immersion objective (100x).

3.3.3.2. Cultural characterization

Cultural characters of *Bacillus* isolates were studied on nutrient agar medium. Colony characters like size, shape, elevation and surface were recorded.

3.4. Screening for functional efficiency under in vitro

Nine *Trichoderma* and five *Bacillus* isolates were screened for various plant growth promoting traits such as IAA, phosphate solubilisation, HCN, and siderophore production.

3.4.1. Screening of isolates for IAA production

Trichoderma and *Bacillus* isolates were screened for the production of IAA (Brick *et al*., 1999). Medium containing Luria Bertani (LB) for *Bacillus* and Potato Dextrose Agar (PDA) for *Trichoderma*, supplemented with 0.06 per cent sodium dodecyl sulphate (SDS) and 1 % glycerol were prepared. Molten and cooled agar

medium was poured into sterile Petri plates. Overnight cultures of *Bacillus* and five days old *Trichoderma* isolates were spot inoculated on the LB agar medium and PDA medium respectively. Immediately sterile discs of Whatman No. 1 filter paper (2 cm × 2 cm) were overlaid and kept for five days incubation at 37 °C. After the incubation period, filter paper discs were removed and soaked with Salkowski's reagent. Isolates producing IAA were identified by the characteristic production of pink to red halo colour around the filter paper.

3.4.1.1. Quantitative estimation of IAA production

IAA production of the *Trichoderma* and *Bacillus* sp. isolates were analysed (Hartmann *et al.*, 1983). Sterilized nutrient broth in test tube with tryptophan solution (100 μg ml^{-1} broth) was used. The tubes were inoculated with 0.1 ml of the culture suspension and incubated at 28 ± 2 °C on an orbital shaker for 5 days. Uninoculated tubes were kept as control. Three replications were maintained for each isolate. After incubation, one millilitre of the culture was transferred aseptically into eppendorf tubes and centrifuged at 8000 rpm for 8 minutes. The supernatant was used for the detection of IAA production. One ml of the supernatant and 4 ml of the reagent were mixed thoroughly. The tubes were incubated for 30 minutes to allow the colour development. The OD values were recorded at 530 nm using spectrophotometer (Perkin Elmer). The IAA production by the cultures was expressed as μg ml^{-1} IAA produced after 48 h.

3.4.2. Screening of isolates for HCN production

Isolates were screened for HCN production using the protocol suggested by Bakker and Schippers (1987). *Bacillus* isolates of 48 h old culture was streaked and five days old *Trichoderma* disc were inoculated on NA medium and PDA medium respectively, supplemented with 4.4 gL^{-1} glycine. Immediately, sterile Whatman No. 1 filter paper soaked with 4 per cent picric acid solution was placed on the upper lid of each plates and incubated at 37 °C for five days. HCN producing isolates were selected based on the changes in the colour of the filter paper. Based on the intensity of the colour, isolates were rated as follows:

Yellow to light brown : Weak (+)

Brown : Moderate (++)

Reddish-brown : Good (+++)

3.4.3. Screening of isolates for siderophore production

Isolates were screened for siderophore production using Chrome Azurol S (CAS) agar medium (Schwyn and Neilands, 1987). Both *Bacillus* and *Trichoderma* isolates were inoculated on CAS agar medium and allowed for incubation at 37 °C for seven days. Isolates which produced halo zone around the colony were positive for siderophore production.

3.4.4. Screening of isolates for ammonia production

Isolates were screened for ammonia production using a method suggested by Cappuccino and Sherman (1992). *Bacillus* isolate of 48 h old culture and seven days old *Trichoderma* cultures were inoculated into test tubes containing 10 ml of 4 per cent peptone water and incubated at room temperature. After incubation, ammonia production was observed by adding 0.5 ml of Nessler's reagent into each test tube. Development of brown colour indicated the ammonia production by the isolates. Based on the intensity of the colour, the isolates were classified as follows:

Yellow : Weak (+)

Orange: : Moderate (++)

Brown : Good (+++)

3.4.5. Screening of isolates for phosphate solubilisation

Phosphate solubilisation ability of different *Bacillus* and *Trichoderma* isolates were studied using Pikovyskaya's agar (Nguyen *et al.*, 1992). The isolates were spotted on Pikovskaya's agar plate and incubated at 28 °C for seven days. Uninoculated plates served as control. The plates were examined and the diameter of halozone and colony diameter were recorded.

$$\text{Solubilisation index (\%)} = \frac{\text{Solubilisation diameter (mm)}}{\text{Colony diameter (mm)}} \times 100$$

3.4.5.1. Quantitative estimation of phosphate solubilisation ability

To the test tubes containing 5 ml of the Pikovyskaya's broth, 2 mg of tri calcium phosphate was added (King, 1932). Tubes were inoculated with 0.1 ml of culture suspension. Uninoculated tube served as control. Tubes were incubated at 28 ± 2 °C for 5 days on orbital shaker. Then the tubes were centrifuged at 8000 rpm for 10 m. Clear solution of 1 ml from the supernatant was transferred to 50 ml volumetric flask along with 10 ml of chloromolybdic acid and the volume was made up to 45 ml. To this, 5 drops of stannous chloride was added. The volume was made up to 50 ml. Immediately, OD values were recorded at 660 nm using spectrophotometer. The OD values were plotted on a standard graph obtained by measuring the absorbance of solution containing known concentration of KH_2PO_4. The pH of the supernatant was also recorded after the incubation period to observe the production of organic acids. Solubilized phosphate quantity was expressed in µg P ml^{-1}.

3.5. *In vitro* screening of isolates for antagonistic activities against pathogens

3.5.1. *In vitro* screening of *Trichoderma* isolates against *Rhizoctonia solani* and *Pythium aphanidermatum*

Trichoderma isolates were screened for antagonistic activity against two major soil borne pathogens of cowpea *viz., Rhizoctonia solani* and *Pythium aphanidermatum* by dual culture method (Dennis and Webster, 1971). Seven day old *Trichoderma* isolates (10 mm discs) and pathogens (10 mm discs) were placed in the PDA medium, leaving 2.5 cm from the margin of the Petri plate. Plates were incubated for 2-7 days at room temperature for development of inhibition zone. Plates containing pathogen alone served as control.

Radial growth of the fungal pathogens was measured, when growth of the pathogen in control plate reached full growth. The per cent inhibition was calculated using the formula,

Per cent inhibition (%) = $\frac{C - T}{C} \times 100$

C – Distance of fungal growth from the point of inoculation to the colony margin in control plate

T – Distance of fungal growth from the point of inoculation to the colony margin in the direction of antagonist.

3.5.2. *In vitro* screening of *Bacillus* isolates against *Rhizoctonia solani* and *Pythium aphanidermatum*

Bacillus isolates (48 h) were inoculated as a line streak and the mycelial discs of the pathogens (10 mm) were inoculated, leaving 2.5 cm from the margin of the Petri plate. Plates were incubated for 2-7 days at room temperature for development of inhibition zone. Plates containing pathogen alone served as control.

Radial growth of the fungal pathogens was measured when growth of the pathogen in control plate reached full growth. The per cent inhibition was calculated using the formula,

Per cent inhibition (%) = $\frac{C - T}{C} \times 100$

C – Distance of fungal growth from the point of inoculation to the colony margin in control plate

T – Distance of fungal growth from the point of inoculation to the colony margin in the direction of antagonist.

3.6. Screening *Bacillus* sp. for biofilm production

All the five isolates of *Bacillus* sp. were screened for biofilm production under *in vitro* condition. Three different methods were employed for screening *Bacillus* sp. for biofilm production.

3.6.1. Test tube method

A loopful of 24 h old culture of *Bacillus* sp. was inoculated to Trypticase Soy broth (10 ml) supplemented with 1% glucose in test tubes and kept for incubation at 37 °C for 24 h (Deka, 2014). After incubation, the tubes were decanted and washed with phosphate saline buffer (NaCl (80 g), KCl (2 g), Na_2HPO_4 (21.7 g), KH_2PO_4 (2.5 g), Distilled water (1L), pH -7.3) and air-dried. Dried tubes were stained with 0.1 % crystal violet. The tubes were washed with deionized water to remove excess crystal violet stain and kept in an inverted position until the tubes dried. Biofilm formation was observed with the presence of visible lined film on the wall of the tube and at the bottom of the test tube.

3.6.2. Congo red agar method (CRA)

Bacillus sp., which formed biofilm, were further confirmed based on the colony morphology on congo red medium (Mathur *et al.*, 2006). The isolates were streaked on Muller Hilton agar medium (HIMEDIA) supplemented with 0.8 g L^{-1} of congo red dye and plates were kept for 48 h at 37 °C. Biofilm forming colonies were identified based on the production of black coloured colonies with dry crystalline consistency on the medium. Weak biofilm producers were pink colonies with no black and dry crystalline colour.

3.6.3. Tissue culture plate method (TCP)

The isolates of *Bacillus* sp. were further confirmed for biofilm production. A loopful of 12 h overnight *Bacillus* culture was inoculated to trypticase soy broth (TSB) supplemented with 1 % glucose and kept for 18 h incubation at 37 °C under stationary condition (Mathur *et al.*, 2006). After incubation, the contents were diluted as 1:100 with fresh medium. Diluted cultures of 0.2 ml aliquots were added to individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plates. TSB added wells without culture served as control.

The loaded tissue culture plates were kept for 24 h incubation at 37 °C. After incubation, contents of each well was decanted and washed four times with 0.2 ml phosphate saline buffer of pH 7.2, to remove planktonic bacteria. Biofilm formed by

adherent bacteria was fixed with sodium acetate (2 %) and stained with crystal violet (0.1 %). Deionized water was used to remove excess stain and the plates were kept for drying. Crystal violet stained the adherent biofilm all along the sides of the well. Optical density (OD) of the adherent stained biofilm was measured using micro ELISA auto-reader at 570 nm wavelength. The OD values directly indicated the intensity and the quantity of the biofilm produced.

3.7. *In vitro* screening of mutual compatibility between fungal and bacterial isolates

3.7.1. Mutual compatibility between *Trichoderma* sp. and *Bacillus* sp. isolates under *in vitro*

Selected isolates of *Trichoderma* and *Bacillus* were screened for mutual compatibility (Dennis and Webster, 1971). For the evaluation, 8 mm disc of *Trichoderma* isolate was inoculated at the centre of the PDA agar plates. *Bacillus* isolates were streaked on both the sides of the disc, leaving 2.5 cm from the margin of the plates. The plates were kept for incubation at 28±2 ^{0}C for 3-5 days and observed daily for any type of inhibition zone. Absence of any inhibition zone indicated compatible ability of isolates.

3.7.2. Screening of mutual compatibility between *Trichoderma* sp. and *Rhizobium* sp. isolates under *in vitro*

Selected isolates of *Trichoderma* were tested for mutual compatibility with *Rhizobium* sp. (Dennis and Webster, 1971). For the evaluation, 8 mm disc of *Trichoderma* isolate was inoculated at the centre of the PDA agar plates (Dennis and Webster, 1971). *Rhizobium* sp. streaked on both the sides of the disc, leaving 2.5 cm from the margin of the plates. The plates were kept for incubation at 28±2 ^{0}C for 3-5 days and observed daily for any type of inhibition zone. Absence of any inhibition zone indicated compatible ability of isolates.

3.7.3. Screening of mutual compatibility between *Bacillus* sp. and *Rhizobium* sp. isolates under *in vitro*

3.7.3.1. Cross streaking method

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The *Bacillus* isolates were studied for their mutual compatibility with *Rhizobium* sp. by cross streak method (Raja *et al.*, 2006). To test the compatibility, *Bacillus* isolates were streaked at one end of the Petr plate as single streak and *Rhizobium* sp. was streaked vertically to this and plates were incubated at incubation at 28±2 ^{0}C for 3-5 days and observed daily for any type of inhibition zone. Three such replications were maintained in the same plate.

3.8. Standardization of carrier material for *Trichoderma* based *Bacillus* biofilm inoculant production

3.8.1. *Trichoderma* based *Bacillus* biofilm production in culture media

In vitro production of *Trichoderma* based biofilm inoculant was carried out using the procedure described by Triveni *et al.* (2012). *Trichoderma* isolates (7 days old) and *Bacillus* isolates (48 h old) were used for the biofilm production. For the preparation of biofilm, 3 ml each of *Trichoderma* sp. and *Bacillus* sp. grown in potato dextrose broth and nutrient broth respectively were inoculated to 100 ml Pikovskaya's broth supplemented with 1 % calcium carbonate. The inoculated flasks were incubated under static and shaking condition with 50 rpm at 30 °C for 16 days.

3.8.2. Selection of carrier material for biofilm based inoculant production

For standardization of carrier material for *Trichoderma* based *Bacillus* biofilm inoculant production, compost, vermiculite, vermicompost, compost: vermicompost (1:1), talc powder were used. The produced biofilm inoculant (300 ml) was mixed with each of the sterilized carrier material (1 kg). It was then air dried to make the final moisture content of 25-30 % (Zaidi *et al.*, 2014). Population of both *Trichoderma* and *Bacillus* in different carrier based formulations were recorded at monthly interval using serial dilution and plate count technique. Carrier based biofilm inoculant with higher *Trichoderma* and *Bacillus* population at 3 MAI was selected for further studies.

3.9. Evaluation of biofilm-based inoculant for the management of soil borne diseases and growth promotion in cowpea under pot culture studies

Three most efficient and compatible *Trichoderma* based *Bacillus* biofilm formulations were screened for their efficiency in the management of *Rhizoctonia solani* and *Pythium aphanidermatum* and also growth promotion in cowpea.

Variety : Bhagyalekshmy

Design : CRD

Replication : 3 (5 plants / replication)

Time of application of treatment: At the time of sowing

Treatment details:

T_1: Biofilm based inoculant-1 (*Trichoderma* based *Bacillus* sp. biofilm-1)

T_2: Biofilm based inoculant -2 (*Trichoderma* based *Bacillus* sp. biofilm -2)

T_3: Biofilm based inoculant -3 (*Trichoderma* based *Bacillus* sp. biofilm -3)

T_4 : *Bacillus* sp. (KAU ref. culture)

T_5 : *Trichoderma* sp. (KAU ref. culture)

T_6 : *Bacillus* sp. + *Trichoderma* sp.

T_7 : Carbendazim + Mancozeb (2 g l^{-1} as soil drenching)

T_8 : Package of Practices, recommendations of KAU (KAU, 2011)

T_9 : PGPR Mix – 2 @ 2.5 kg ha^{-1}

T_{10} : Absolute control

Organic package (Adhoc) (KAU, 2009) was applied for all the treatments except T_8 and T_{10}, which included seed treatment with *Rhizobium* @ 0.5 kg 10 kg^{-1} of seeds, manuring with FYM @ 20 t ha^{-1} and lime application @ 250 kg ha^{-1}. All the treatments were supplemented with FYM or cowdung @ 2 t ha^{-1} along with rock phosphate @ 100 kg ha^{-1}.

Bacillus sp. @ 4 g kg^{-1} seed and *Trichoderma* sp. @ 4 g kg^{-1} seed were applied at the time of seed treatment. The plants were challenge inoculated with each pathogen in two separate sets of experiments. Challenge inoculation of *Rhizoctonia solani* (Plate 19) and *Pythium aphanodermatum* (Plate 20) @ 20 g plant^{-1} (@ 8×10^5 cfu per g) was done one month after the application of antagonists. *Pythium aphanidermatum* was grown on sterilized carrot bits for 5 days and the fully grown *Pythium aphanidermatum* was used for the artificial inoculation (Plate 20).

3.10. Observations

Observations on germination percentage, plant height (cm), number of leaves, days taken for flowering, fresh and dry weight of plant (g), percent incidence of root-rot and collar-rot diseases, per cent pest infestation, yield per plant (g) were recorded at fortnightly intervals.

Nutritional content of the potting mixture *viz.,* pH, CEC, organic carbon, nitrogen, phosphorus and potassium content before and after planting were recorded. Population count of *Trichoderma* and *Bacillus* in potting mixture (cfu g^{-1}) were also recorded using standard protocols.

3.10.1. Germination percentage

$$\text{Germination percentage} = \frac{\text{No. of plants germinated}}{\text{Total no. of plants per treatment}} \times 100$$

3.10.2. Plant height

The distance from the base of the plant to the tip was taken as plant height and expressed in centimetres. Observations were recorded at 15 days intervals.

3.10.3. Number of leaves

Number of leaves per plants were recorded at 15 days intervals.

3.10.4. Days taken for flowering

Number of days taken for first flowering was recoded.

3.10.5. Fresh and dry weight of the plant

Fresh and dry weight of the plant were recorded after harvesting and expressed in gram per plant.

3.10.6. Per cent disease incidence of collar-rot and root-rot disease

Per cent disease incidence of collar-rot and root-rot disease were recorded as when noticed.

3.10.7. Pest infestation

Per cent pest infestation were recorded throughout the crop period.

3.10.8. Enumeration of inoculated *Trichoderma* sp. and *Bacillus* sp. in potting mixture (cfu per g)

Population count of *Trichoderma* and *Bacillus* in potting mixture was recorded using serial dilution plate technique (Johnson and Curl, 1972). The collected potting mixture was serially diluted from 10^{-1} to 10^{-6} dilutions. One millilitre suspension of the dilutions were poured into plates containing PDA and NA for *Trichoderma* and *Bacillus* respectively. After solidification, plates were kept for 2-5 days incubation. Enumeration of *Trichoderma* and *Bacillus* were done using serial dilution and plate count technique.

3.10.9. Analysis of nutrient content in the potting mixture

Nutrient status of the potting mixture like pH, CEC, organic carbon, nitrogen, phosphorus and potassium were recorded before and after the pot culture experiment.

3.10.9.1. Soil Reaction (pH)

The pH of the potting mixture was determined in 1:2.5 sample-water suspensions. Ten gram of 2 mm sieved air dried potting mixture was taken in a 50 ml beaker with 25 ml distilled water. It was stirred well and kept for half an hour. Again, it was stirred and reading was taken using pH meter (ELICO L1 120).

3.10.9.2. Electrical conductivity (EC)

The electrical conductivity of potting mixture was determined (Rayment and Higginson, 1992). Ten grams of sample was taken in a 50 ml beaker to which 25 ml distilled water (1:2.5 ratio) was added. Suspension was stirred well at regular intervals for 30 minutes and electrical conductivity was recorded using EC meter

3.10.9.3. Organic Carbon content

Soil organic carbon was determined using Walkley and Black wet digestion method (Walkley and Black, 1934). The per cent organic carbon was calculated by the equation:

$$OC\ (\%) = \frac{(\text{meq } K_2Cr_2O_7 - \text{meq } Fe(NH_4)_2SO_4)\ 0.003 \times 100 \times 1.3}{\text{Weight of soil (g)}}$$

3.10.9.4. Available nitrogen content

The available nitrogen content of the soil was recorded before and after the pot culture experiment (Subbiah and Asija, 1956). The available nitrogen content was calculated using the equation:

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

Where, TV= Titre value

BV = Blank value

N = Normality of H_2SO_4

Y = Weight of C source

3.10.9.5. Available phosphorus content

The concentration vs. absorbance curve was plotted on a graph paper (Bray, 1948). The available phosphorus content was calculated using the equation:

$$\text{Available P (mg/kg soil)} = \frac{\text{Absorbance for sample}}{\text{Slope of the standard curve}} \times \frac{50}{5} \times \frac{25}{5}$$

3.10.9.6. Available potassium content

Estimation of available potassium was done by flame photometric method (Jackson, 1973). After attaching the appropriate filter and adjusting the gas and air pressure, the reading was set in the flame photometer as zero for the blank (potassium chloride) and at 100 for 20 µg/ml of potassium. The curve was obtained by plotting the readings against the different concentrations (5, 10, 15 and 20 µg/ml) of K.

$$\text{Available K (mg kg}^{-1}\text{ soil)} = \mu\text{g K per ml of aliquot} \times \frac{25}{5}$$

3.11. Molecular characterization of selected isolates

3.11.1. Sequence analysis of selected isolates

The bacterial isolates were identified by16S rDNA sequencing

3.11.1.1 Amplification of 16S rDNA gene

Using micropipette, single colony of the isolate was mixed with 10 µl of sterile water. 2µl of this suspension was used as template for amplification of 16S rRNA gene. The details of primers (Siddapura *et al,* 2010) used are given in Table 2.
Polymerase chain reaction was carried out in Eppendorf Master Cycler (Gradient) using PCR master mix 'Emerald Amp GT PCR'. The composition of the reaction mixture for PCR is given in as given below.

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

Primer details	Sequence 5'- 3'	Length in bp
8 F	AGAGTTTGATCCTG GCTCAG	20
1522 R	AAG GAG GTG ATC CAG CCG CA	20

Composition of PCR reaction mixture

Component	Per reaction volume required
Master mix	12.5 µl
Template	2.0 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
dH2O	9.5 µl
Total	25.0 µl

Details of master cycle programme

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

7	Final hold	4	10.00

3.11.1.2. Agarose gel electrophoresis

The quality of isolated DNA was evaluated though agarose gel electrophoresis (Sambrook *et al.*, 1989). 100 ml of 1X TAE buffer was prepared from the 50X TAE (pH 8.0) stock solutions. 1.0 g of agarose (Genei, Low EEO) was added to the 1X TAE buffer in conical flask. Agarose was dissolved in buffer by heating and cooled to 42-450C. Ethidium bromide prepared from a stock of 10 mg ml^{-1} was added to it at a concentration of 0.5 μg ml^{-1} and mixed well without the formation of bubbles. After wiping the gel casting tray and comb with alcohol, the comb was placed properly in the casting apparatus. Prepared agarose was poured into the tray and left for solidification for 30-45 minutes. To make the well, the comb was pulled out and gel was placed in the buffer tank containing 1X TAE buffer with well side directed towards the cathode. 2 μl of the PCR product mixed with gel loading dye was carefully loaded into the wells using a micro pipette. The Gene Ruler 1 kb DNA ladder was used as the molecular weight marker. The cathode and anode of the electrophoresis unit were connected to the power pack and the gel was run at constant voltage of 100 V. The power was turned off when the tracking dye reached at about 3 cm from the anode end.

3.11.1.3. Gel documentation

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

3.11.1.4. Purification and sequencing of PCR product

The PCR product was purified and sequenced at Vision Scientific Services Angamaly, using the primers 8F and 1522r.

3.11.1.5. Nucleotide sequence analysis

The Base Local Alignment Search Tool for Nucleotides (blastn) programme (http: //blast.ncbi.nlm.nih.gov/Blast) was used to find out the homology of the nucleotide sequences

4. RESULTS

A study was conducted on "Evaluation of biofilm based microbial antagonists for the management of soil borne diseases and growth promotion in cowpea (*Vigna unguiculata* L. Walp)" in order to evaluate a novel biofilm based formulation and its effect on disease management and growth in cowpea. The results obtained from the experiments are detailed in this chapter.

4.1. Population of the *Trichoderma* sp. and *Bacillus* sp. in rhizosphere soil samples

Rhizosphere soil samples were collected from ten different cowpea growing areas of Thrissur district with six samples from healthy plants and four from collar rot disease infected cowpea (Table 1) (Plate 1). Among the different samples, Chellakara (TCH) recorded highest population of *Trichoderma* sp. (4.8×10^3 cfu g^{-1}) followed by Pazhayanur (TPZ) (3.7×10^3 cfu g^{-1}) (Table 2). The lowest *Trichoderma* sp. population was recorded in Chalakudy (TCK) (1.8×10^3 cfu g^{-1}).

Population of the *Bacillus* sp. were highest in Pananchery (BPN) (4.48×10^5 cfu g^{-1}) followed by Mattathur (BMT) (3.24×10^5 cfu g^{-1}). The lowest *Bacillus* sp. population was recorded in Pananchery (BPN) (2.17×10^5 cfu g^{-1}).

4.2. Morphological and cultural characters of *Trichoderma* sp.

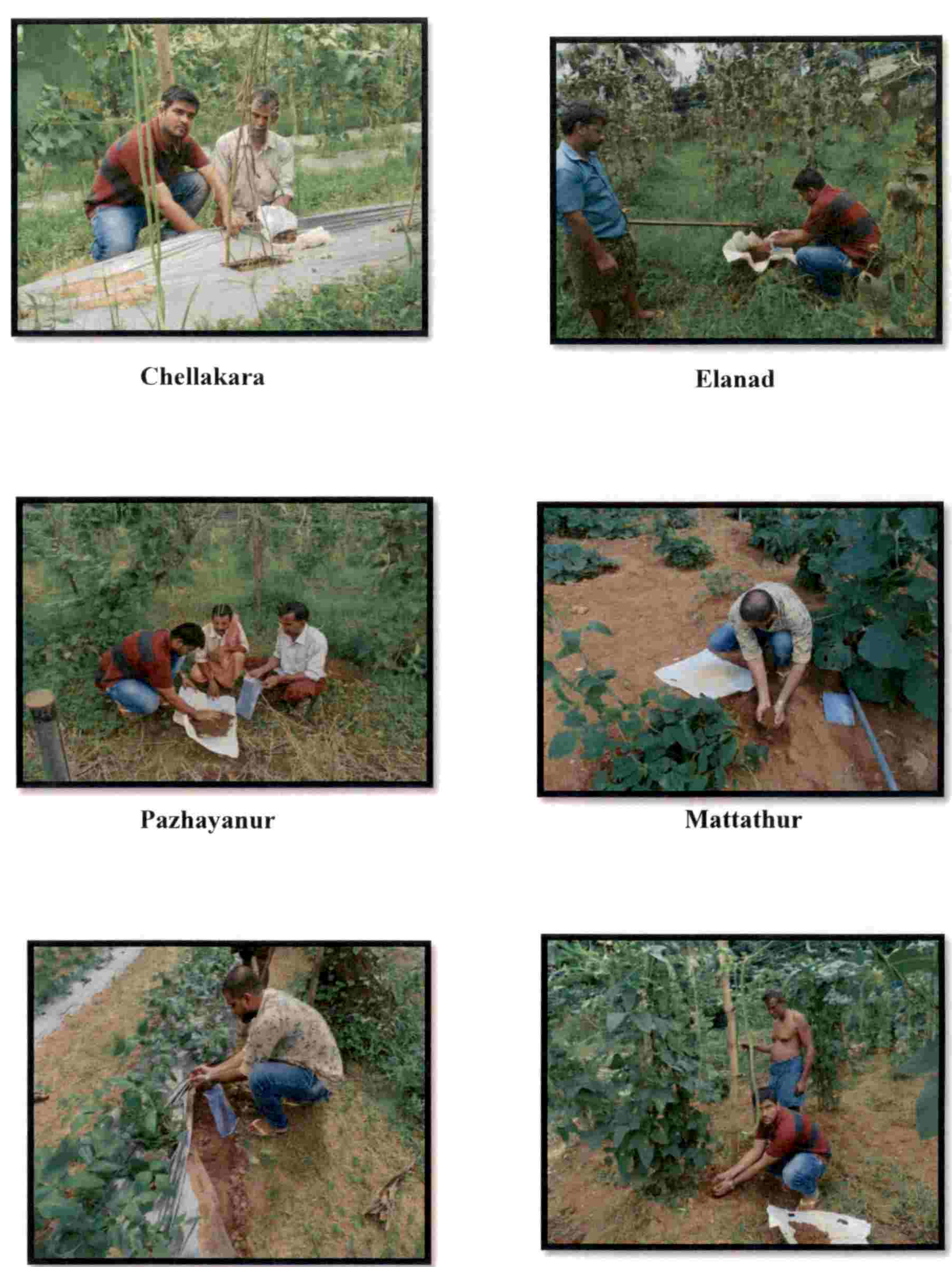
Nine *Trichoderma* sp. were obtained (Table 3 and Plate 2). The colonies were smooth surfaced and colour changed from whitish green to dark green. The hyphae were septate and hyaline. Conidiophores were hyaline and conidia green in colour. Hence, the isolates were tentatively identified as *Trichoderma* sp. based on the standard keys. (Shah *et al*., 2012) (Table 4 and Plate 4).

4.3. Morphological and cultural characters of *Bacillus* sp.

The five isolates (Plate 3) from all the locations were gram positive, long rods and endospore positive (Plate 4). These characters were compared with the standard keys as described in Bergey's Manual of determinative Bacteriology (Holt *et al.,* 2004) and tentatively assigned the isolates to genus *Bacillus* sp. (Table 5).

Table 1. Rhizosphere soil samples collected from different cowpea growing areas of Thrissur district

Location	Healthy / diseased	Geographical position		
		Latitude (^{0}N)	Longitide (^{0}E)	Elevation (ft)
Chellakara	Healthy	10.66938	076.35297	205
Pazhayanur	Healthy	10.66219	076.41782	172
Nadathara	Healthy	10.503476	076.280601	150
Pananchery	Healthy	10.66076	076.42987	170
Vellanikkara	Healthy	10.548584	076.28609	182
Elanad	Healthy	10.62417	076.38636	272
Chalakudy	Diseased	10.31319	076.34290	83
Mattathur	Diseased	10.36185	076.38124	121
Mala	Diseased	10.24862	076.27211	24
Mullassery	Diseased	10.54026	076.08824	76



Chellakara

Elanad

Pazhayanur

Mattathur

Mala

Pananchery

Plate 1. Cowpea rhizosphere soil sample collection from different locations of Thrissur district

Table 2. Population of *Trichoderma* sp. and *Bacillus* sp. in cowpea rhizosphere soil

Location	***Trichoderma* sp. (x10^3 cfu g^{-1} of soil)**	***Bacillus* sp. (x10^5 cfu g^{-1} of soil)**
Chellakara (CH)	4.8^{a} (3.68)	2.66 (3.42)
Pazhayanur (PZ)	3.7^{ab} (3.56)	0.00 (0.71)
Chalakudy (CK)	1.8^{c} (3.25)	0.00 (0.71)
Mattathur (MT)	3.23^{abc} (3.50)	3.24 (3.51)
Mala (ML)	2.56^{bc} (3.40)	2.17 (3.33)
Mullasery (MS)	3.68^{ab} (3.56)	2.84 (3.45)
Elanad (EL)	0.00 (0.71)	0.00 (0.71)
Nadathara (NT)	0.00 (0.71)	0.00 (0.71)
Pananchery (PN)	0.00 (0.71)	4.48 (3.65)
Vellanikara (VL)	0.00 (0.71)	0.00 (0.71)
CD (0.05)	1.819	NS

NS- Non significant

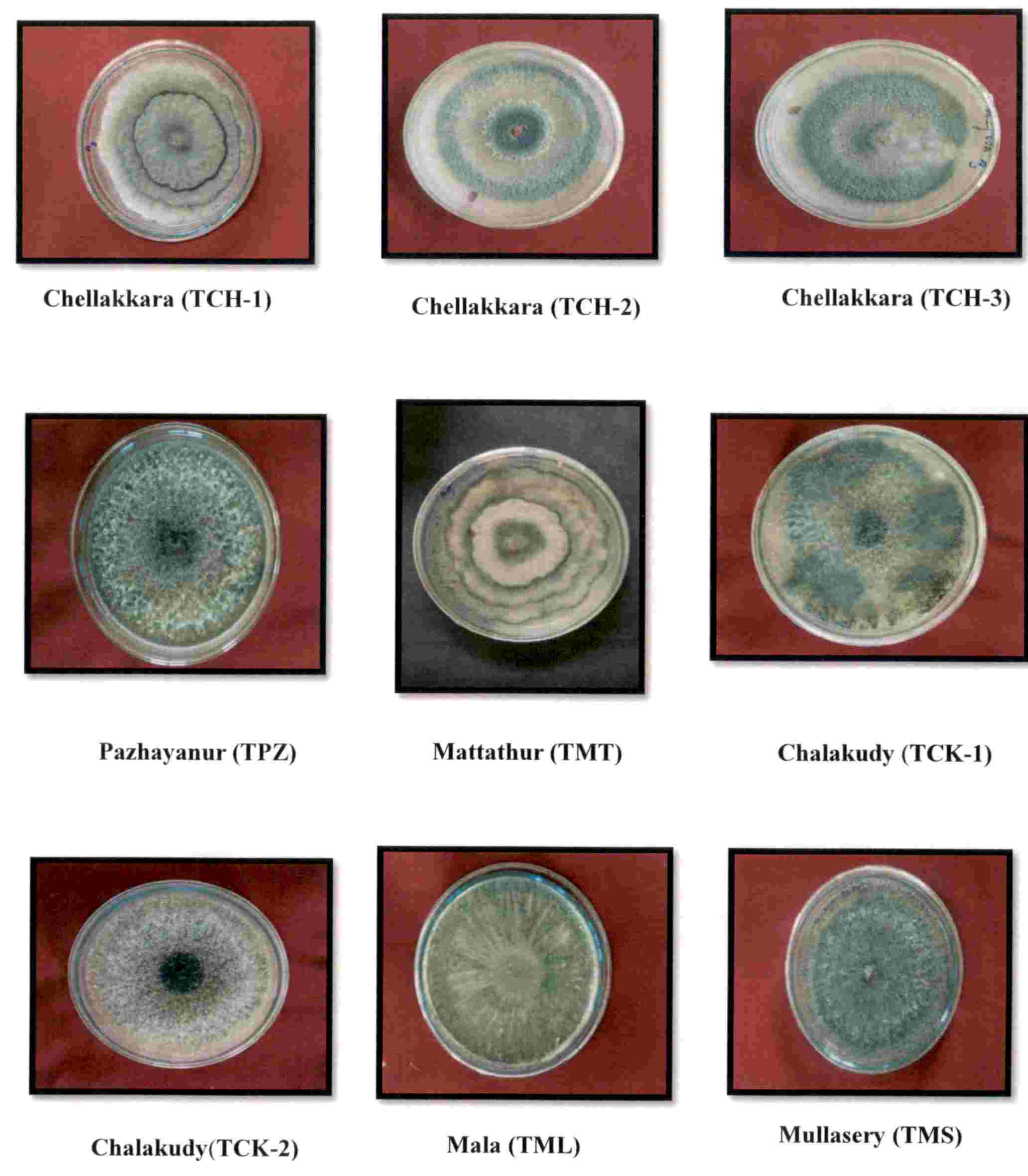
Each value represents the mean of three replications

Values in the parenthesis indicate log transformed values

Table 3. Morphotypes of *Trichoderma* sp. and *Bacillus* sp. obtained from rhizosphere soil of cowpea

Location	Isolate code	
	***Trichoderma* sp.**	***Bacillus* sp.**
Chellakara (CH)	TCH-1, TCH-2, TCH-3	BCH
Mattatur (MT)	TMT	BMT
Mullassery (MS)	TMS	BMS
Mala (ML)	TML	BML
Chalakudy (CK)	TCK-1, TCK-2	-
Pananchery (PN)	-	BPN
Pazhayanur (PZ)	TPZ	-

(–) : Indicate no isolates



Chellakkara (TCH-1) Chellakkara (TCH-2) Chellakkara (TCH-3)

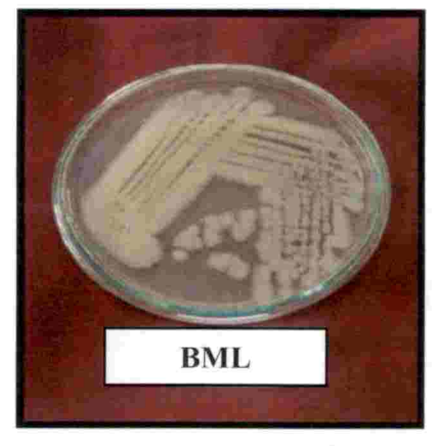
Pazhayanur (TPZ) Mattathur (TMT) Chalakudy (TCK-1)

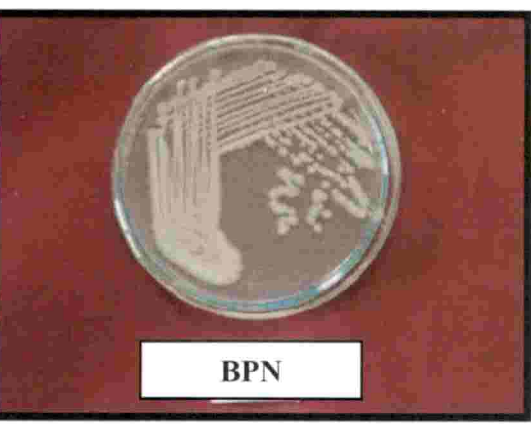
Chalakudy(TCK-2) Mala (TML) Mullasery (TMS)

Plate 2. Different morphotypes of *Trichoderma* sp.

Table 4. Morphological and cultural characterization of *Trichoderma* sp.

Isolates	**Concentric rings (after 5 days)**	**Colour of conidia (after 5 days)**	**Conidia production (after 5 days)**
TCH-1(Chellakara)	2	Light green	Entire medium
TCH-2 (Chellakara)	2	Light green	Entire medium
TCH-3 (Chellakara)	3	Whitish green	Centre
TMT (Mattathur)	1	Dark green	Margin
TPZ (Pazhayanur)	1	Whitish green	Margin
TCK-1(Chalakudy)	1	Dark green	Centre
TCK-2 (Chalakudy)	1	Dark green	Entire medium
TML (Mala)	1	Whitish green	Entire medium
TMS (Mullassery)	2	Whitish green	Enitre medium





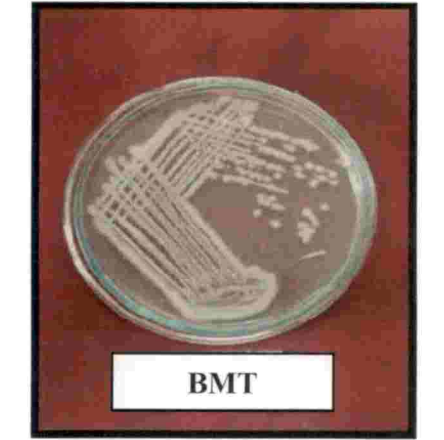
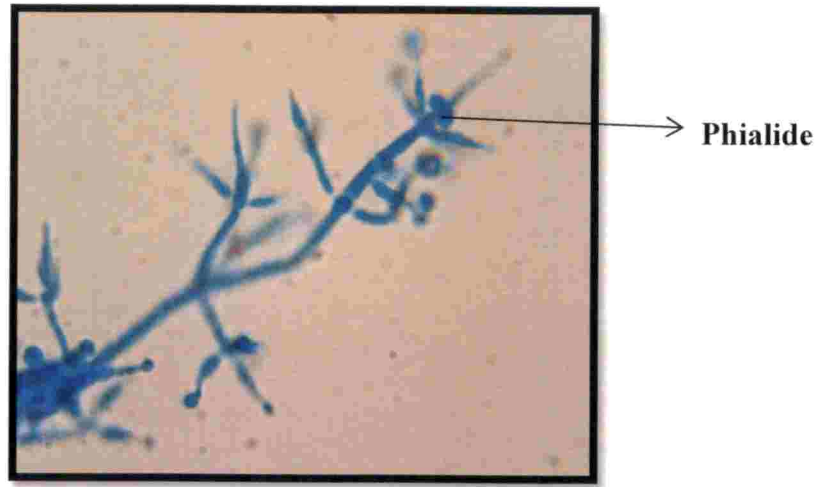
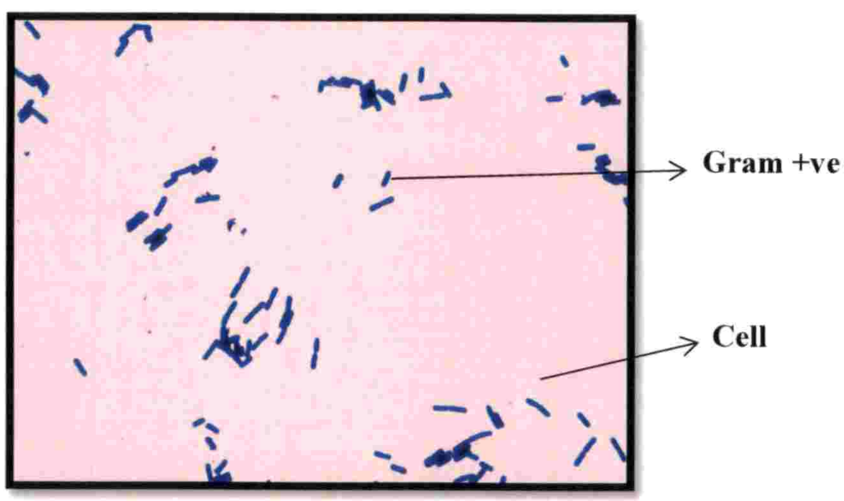


Plate 3. Morphotypes of *Bacillus* sp.



A . *Trichoderma* sp. (TCH)



B. *Bacillus* sp. (BCH)

Plate 4. Microscopic observation of *Trichoderma* sp. and *Bacillus* sp.

Table 5. Morphological, cultural and biochemical characters of *Bacillus* sp.

Isolates	Gram reaction	Shape	Size	Margin	Surface texture	Elevation	Colour	Endospore Staining
BCH (Chellakara)	+ve	Rod	Large	Irregular	Rough	Flat	Whitish cream	+ve
BPN (Pananchery)	+ve	Rod	Large	Irregular	Smooth	Flat	Whitish cream	+ve
BMT (Mattathur)	+ve	Rod	Large	Irregular	Rough	Flat	Cream	+ve
BML (Mala)	+ve	Rod	Large	Irregular	Rough	Flat	Cream	+ve
BMS (Mullassery)	+ve	Rod	Small	Circular	Mucoid	Elevated	Dull white	+ve

4.4. *In vitro* screening of isolates for plant growth promoting (PGP) activities

4.4.1. Screening of *Trichoderma* sp. and *Bacillus* sp. for indole acetic acid (IAA) production

Nine *Trichoderma* sp. and five *Bacillus* sp. isolates were screened for IAA production under *in vitro*. Based on the development of pink to red colour, four *Trichoderma* sp. (TMT, TML, TCH-1and TCK-2) and two *Bacillus* sp. (BCH, BMS) isolates were positive for IAA production.

4.4.1.1. Quantitative estimation of IAA produced by Trichoderma sp. and Bacillus sp.

The isolates which showed positive for IAA production were further confirmed by quantitative estimation of IAA (Plate 5A and 5B). Among *Trichoderma* sp. isolates, TCK-2 was the highest IAA producer (24.03 $\mu g\ ml^{-1}$) followed by TML (14.77 $\mu g\ ml^{-1}$). The lowest IAA production was recorded by TCH-1 (4.25 $\mu g\ ml^{-1}$).

Among *Bacillus* sp., BCH produced highest IAA (6.20 $\mu g\ ml^{-1}$) whereas, isolate BMS produced 5.34 $\mu g\ ml^{-1}$ of IAA (Table 6).

4.4.2. Qualitative screening of *Trichoderma* sp. and *Bacillus* sp. for phosphate solubilisation

Among the *Trichoderma* sp., none of the isolates showed solubilisation zone around the colony.

Among the *Bacillus* sp., BML and BPN were positive for phosphate solubilisation (Table 7) (Plate 6). However, the BPN isolate recorded highest P solubilisation index (29.45 %) followed by BML (23.55 %).

4.4.2.1. Quantitative estimation of phosphate solubilisation by Trichoderma sp. and Bacillus sp.

Though, all *Trichoderma* isolates showed negative result during qualitative screening, four isolates (TMT, TCH-1, TPZ, TMT) recorded phosphate solubilisation

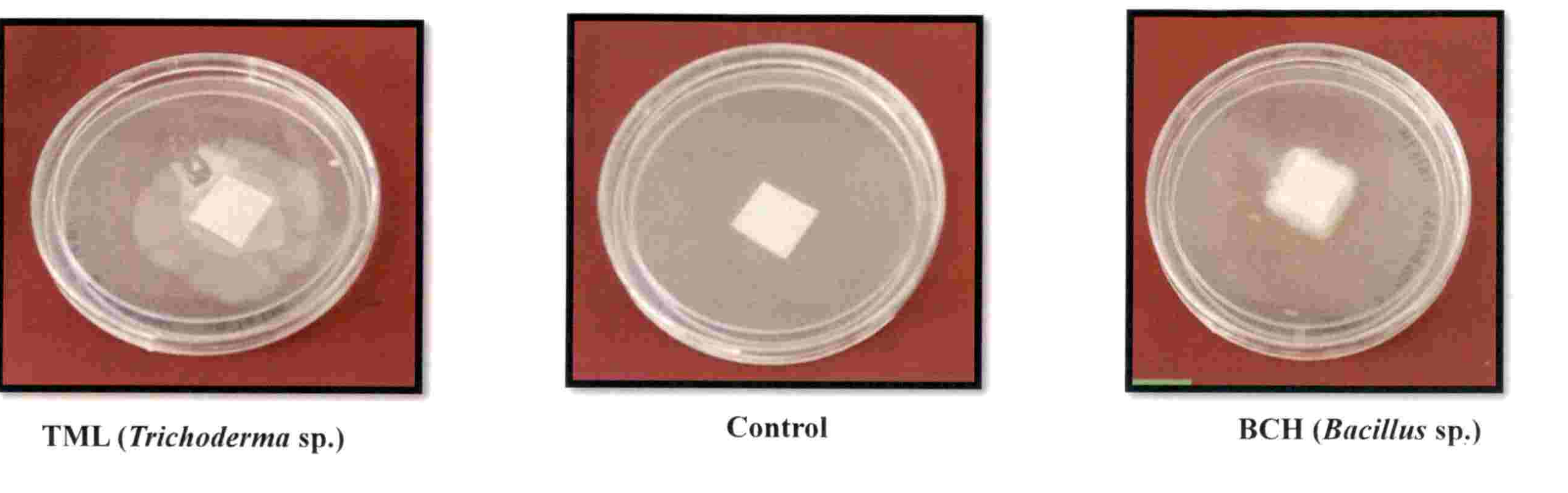


Plate 5A . Qualitative estimation of IAA production by *Trichoderma* sp. and *Bacillus* sp.

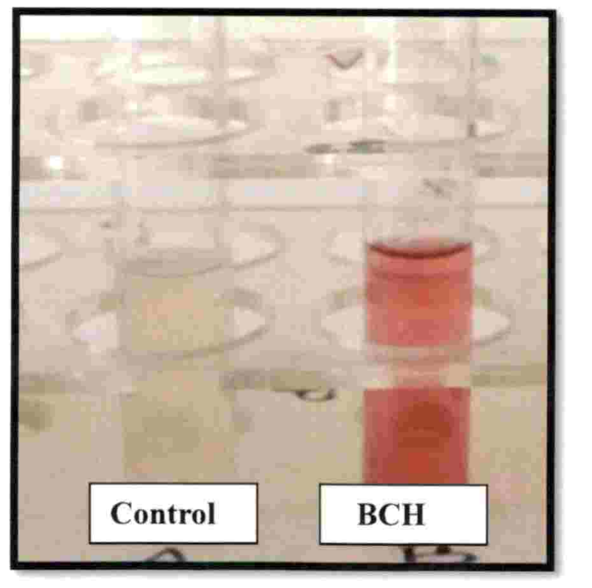


Plate 5B . Quantitative estimation of IAA production by *Trichoderma* sp. and *Bacillus* sp.

Table 6. Screening of *Trichoderma* sp. and *Bacillus* sp. for indole acetic acid (IAA) production under *in vitro*

Isolate	Isolate Code	IAA production (μg ml $^{-1}$)
Trichoderma sp.	TML (Mala)	14.77
	TCH-1 (Chellakara)	4.25
	TMT (Mattathur)	8.14
	TCK-2 (Chalakudy)	24.03
Bacillus sp.	BCH (Chellakara)	6.20
	BMS (Mullassery)	5.34

T: *Trichoderma* sp. B: *Bacillus* sp.

Table 7. Screening of *Bacillus* sp. for phosphate solubilisation on Pikovskaya's agar medium

Isolates	Formation of solubilisation zone	Solubilisation index (%)
BML (Mala)	+	23.55
BMS (Mullassery)	-	-
BPN (Pananchery)	+	29.45
BMT (Mattathur)	-	-

B: *Bacillus* sp.

(+): Indicates solubilisation zone around the colony

(–): Indicates no solubilisation zone around the colony

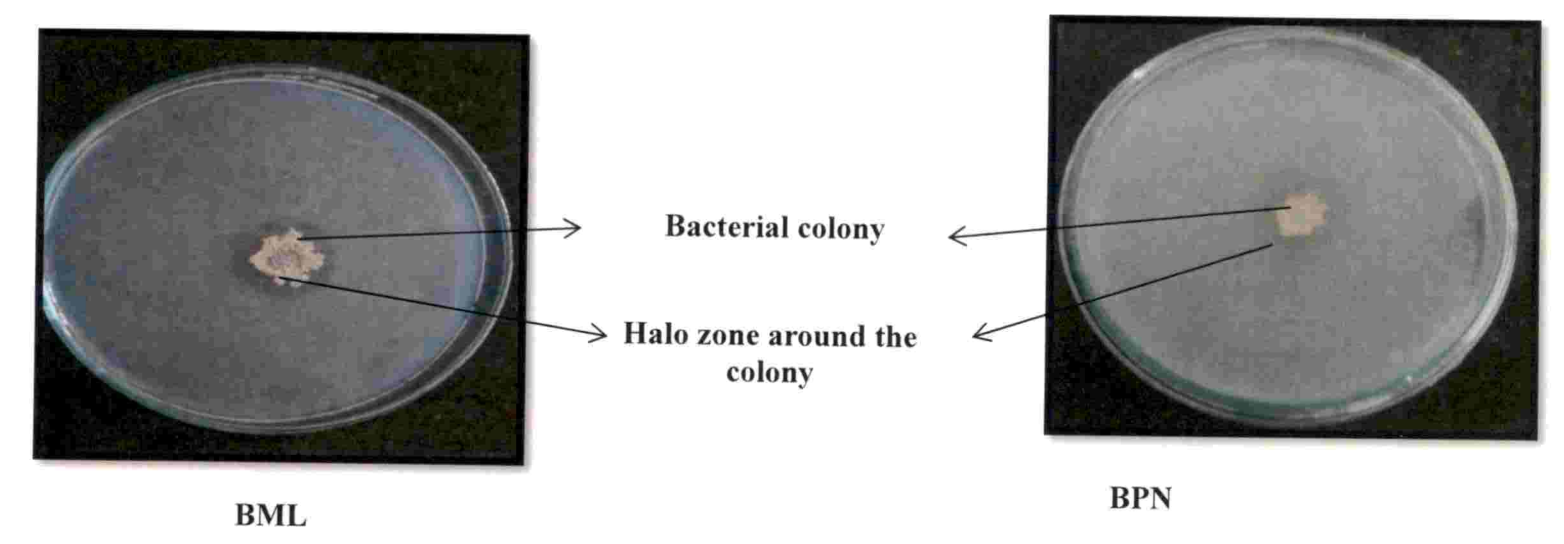


Plate 6. Phosphate solubilization efficiency by *Bacillus* sp.

Under quantitative estimation. However, TPZ recorded the maximum P solubilisation (147.2 μg ml^{-1}) followed by TMT (139.6 μg ml^{-1}) (Table 8).

Among the *Bacillus* sp., BPN recorded the highest P-solubilisation (151.3 μg ml^{-1}) followed by BML which recorded 74.0 μg ml^{-1}

4.5. *In vitro* screening of isolates for antagonistic activities

4.5.1. Screening of *Trichoderma* sp. and *Bacillus* sp. for hydrogen cyanide production

Nine *Trichoderma* isolates were screened for HCN production under *in vitro* and the intensity varied from brown to yellow colour. Two *Trichoderma* sp. isolates (TCH-1 and TMT) were positive for HCN production (Table 9) (Plate 7).

Five *Bacillus* sp. were screened for HCN production under *in vitro* and the intensity varied from brown to yellow colour (Table 9). Two *Bacillus* sp. isolates (BCH and BMS) were positive for HCN production (Plate 7).

4.5.2. Screening of *Trichoderma* sp. and *Bacillus* sp. for ammonia production

Nine *Trichoderma* sp. isolates were screened for ammonia production under *in vitro* and the intensity varied from reddish brown to light brown colour. Only five *Trichoderma* sp. isolates (TCH-1, TMT, TMS, TPZ and TCK-1) were positive for ammonia production (Table 10) (Plate 8).

Similarly, all the five *Bacillus* sp. were screened for ammonia production under *in vitro* and the intensity varied from reddish brown to light brown colour. All the five *Bacillus* sp. isolates (BCH, BML, BMT, BMS and BPN) were positive for ammonia production

4.5.3. Screening of *Trichoderma* sp. and *Bacillus* sp. for siderophore production

Among the five *Bacillus* sp., only two isolates (BPN and BML) were positive for siderophore production (Table 11) (Plate 9). However, none of the *Trichoderma* sp. were positive for siderophore production.

Table 8. Quantitative estimation of phosphate solubilisation by *Trichoderma* sp. and *Bacillus* sp. under *in vitro*

Isolate	Isolate	P- solubilized (µg ml^{-1})	Final pH
Trichoderma sp.	TMT (Mattathur)	139.6	4.4
	TMS (Mullassery)	98.8	4.3
	TPZ (Pazhayanur)	147.2	4.1
	TCH-1 (Chellakara)	86.35	5.2
Bacillus sp.	BPN (Pananchery)	151.3	5.5
	BML (Mala)	74.00	5.7

T: *Trichoderma* sp. B: *Bacillus* sp.

Table 9. Screening of *Trichoderma* sp. and *Bacillus* sp. for hydrogen cyanide (HCN) production under *in vitro*

Isolate	Isolate	HCN production
Trichoderma sp.	TCH_1 (Chellakara)	++
	TPZ (Pazhayanur)	++
Bacillus sp.	BCH (Chellakara)	++
	BMT (Mattathur)	+++

T: *Trichoderma* sp. B: *Bacillus* sp.

Moderate: (++) High: (+++)

Table 10. Screening *Bacillus* sp. and *Trichoderma* sp for ammonia production under *in vitro*

Isolate	Isolate code	Ammonia production
Trichoderma sp.	TCH_1 (Chellakara)	+++
	TMT (Mattathur)	++
	TMS (Mullassery)	+++
	TPZ (Pazhayanur)	++
	TCK_1 (Chalakudy)	++
Bacillus sp.	BCH (Chellakara)	++
	BML (Mala)	+++
	BMS (Mullassery)	+
	BPN (Pananchery)	+
	BMT (Mattathur)	++

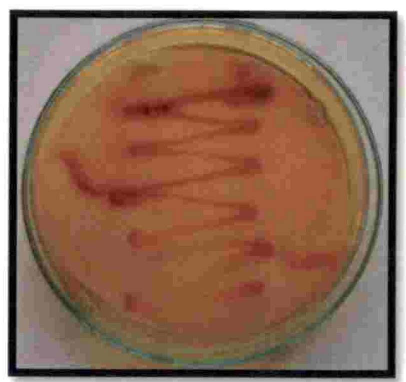
T: *Trichoderma* sp. B: *Bacillus* sp.

Low : (+) Medium : (++) High : (+++)

TMT (*Trichoderma* sp.)

TCH-1 (*Trichoderma* sp.)

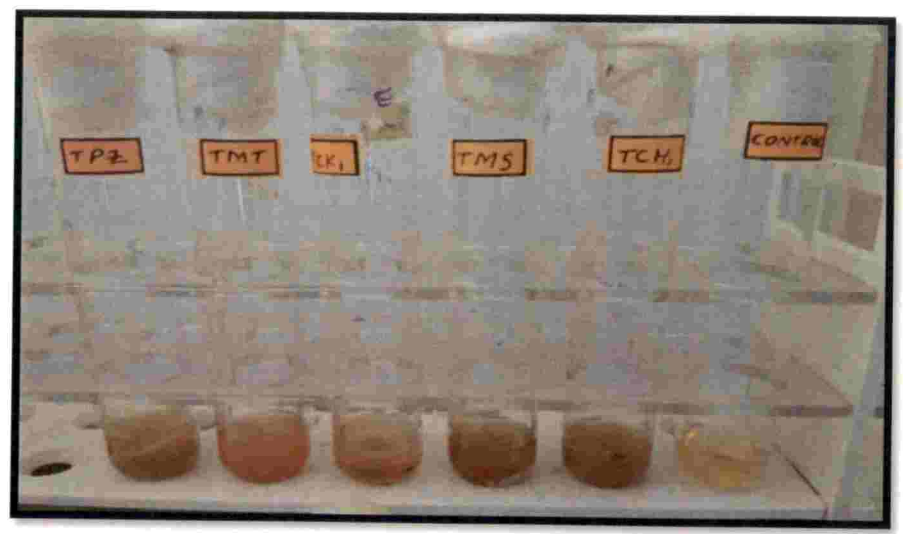
BCH (*Bacillus* sp.)



BMS (*Bacillus* sp.)

Control

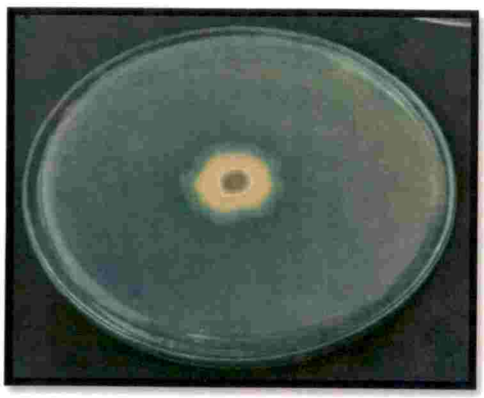
Plate 7. Hydrogen cyanide production by *Trichoderma* sp. and *Bacillus* sp.



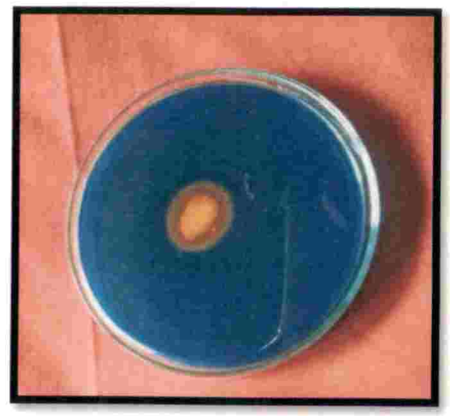
Ammoia production by *Trichoderma* sp.

Ammonia production by *Bacillus* sp.

Plate 8. Ammonia production by *Trichoderma* sp. and *Bacillus* sp.



BML



BPN

Control

Plate 9. Siderophore production by *Bacillus* sp.

Table 11. Screening of *Bacillus* sp. for siderophore production under *in vitro*

	Isolates	Siderophore production
Trichoderma sp.	TCH1 (Chellakara)	-
	TCH2 (Chellakara)	-
	TCH3 (Chellakara)	-
	TPZ (Pazhayanur)	-
	TCK1 (Chalakudy)	-
	TCK2(Chalakudy)	-
	TMT (Mattathur)	-
	TML (Mala)	-
	TMS (Mullassery)	-
Bacillus sp.	BCH (Chellakara)	-
	BMS (Mullassery)	-
	BML (Mala)	+
	BPN (Pananchery)	+
	BMT (Mattathur)	-

(+): Indicates production of yellow-orange halo around the colony

(-): Indicates absence of yellow-orange halo around the colony

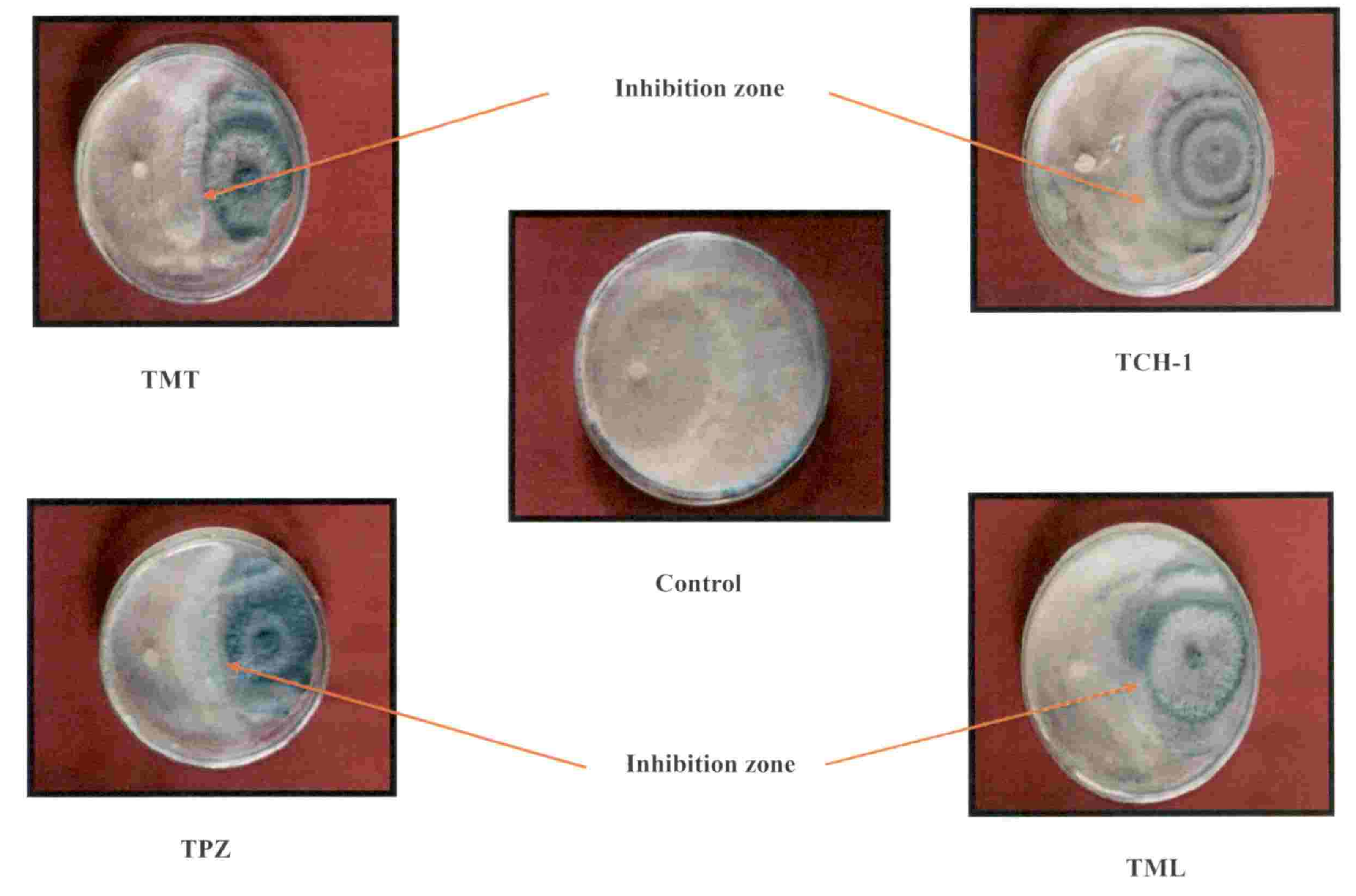


Plate 10. Antagonistic activity of *Trichoderma* sp. against *Rhizoctonia solani*

4.5.4. Screening of *Trichoderma* sp. and *Bacillus* sp. for antagonistic activity against *Rhizoctonia solani*

Trichoderma sp. and *Bacillus* sp. isolates were screened for antagonistic activity against *Rhizoctonia solani* using dual culture plate technique. Among the *Trichoderma* sp., TCH-1 isolate recorded the highest inhibition (51.1 %) followed by TPZ isolate (50.0 %). The lowest per cent inhibition (44.6 %) was recorded by TCK-2 isolate (Plate 10).

Among the five *Bacillus* isolates, none of the isolates showed inhibition against *Rhizoctonia solani* (Table 12) (Plate 11).

4.5.5. Screening of *Trichoderma* sp. and *Bacillus* sp. for antagonistic activity against *Pythium aphanidermatum*

Trichoderma sp. and *Bacillus* sp. isolates were screened for antagonistic activity against *Pythium aphanidermatum.* Among all the *Trichoderma* sp., all isolates recorded antagonistic activity against *Pythium aphanidermatum*. The highest per cent inhibition (57.7 %) was recorded by TCH-1 followed by TML isolate (53.3 %). The lowest inhibition (47.7 %) was recorded by TCH-2 (Plate12).

None of the *Bacillus* isolates showed antagonism against *Pythium aphanidermatum* under *in vitro* (Table 12) (Plate 13).

4.6. Screening of *Bacillus* sp. for biofilm production under *in vitro*

Five isolates of *Bacillus* sp. were screened for biofilm production under *in vitro* using three different methods *viz.,* tube method, congo red agar method and tissue culture plate method (Plate 14). In the tube method, four *Bacillus* isolates (BCH, BMT, BML and BPN) were positive for biofilm production indicating blue colour around the periphery of the wall of the tubes. Under congo red method, only two *Bacillus* isolates (BCH and BMT) were found positive for biofilm production.

The biofilm producers were further confirmed by quantitative screening through tissue culture plate method. The BCH isolate recorded the maximum OD

Table 12. Antagonistic activity of *Trichoderma* sp. against *Rhizoctonia solani* and *Pythium aphanidermatum* under *in vitro*

	Isolates	Per cent inhibition against *Rhizoctonia solani*	Per cent inhibition against *Pythium aphanidermatum*
Trichoderma sp.	TCH1 (Chellakara)	51.1	57.7
	TCH2 (Chellakara)	45.5	47.7
	TCH3 (Chellakara)	47.7	51.1
	TPZ (Pazhayanur)	50.0	51.1
	TCK1 (Chalakudy)	46.6	52.2
	TCK2(Chalakudy)	44.4	49.9
	TMT (Mattathur)	44.6	48.8
	TML (Mala)	48.8	53.3
	TMS (Mullassery)	47.7	51.1
Bacillus sp.	BCH (Chellakara)	-	-
	BMS (Mullassery)	-	-
	BML (Mala)	-	-
	BPN (Pananchery)	-	-
	BMT (Mattathur)	-	-

Each value represents mean of three replications

(–) : No inhibition

No inhibition zone

BML

BCH

Control

Plate 11. Antagonistic activities of *Bacillus* sp. against *Rhizoctonia solani*

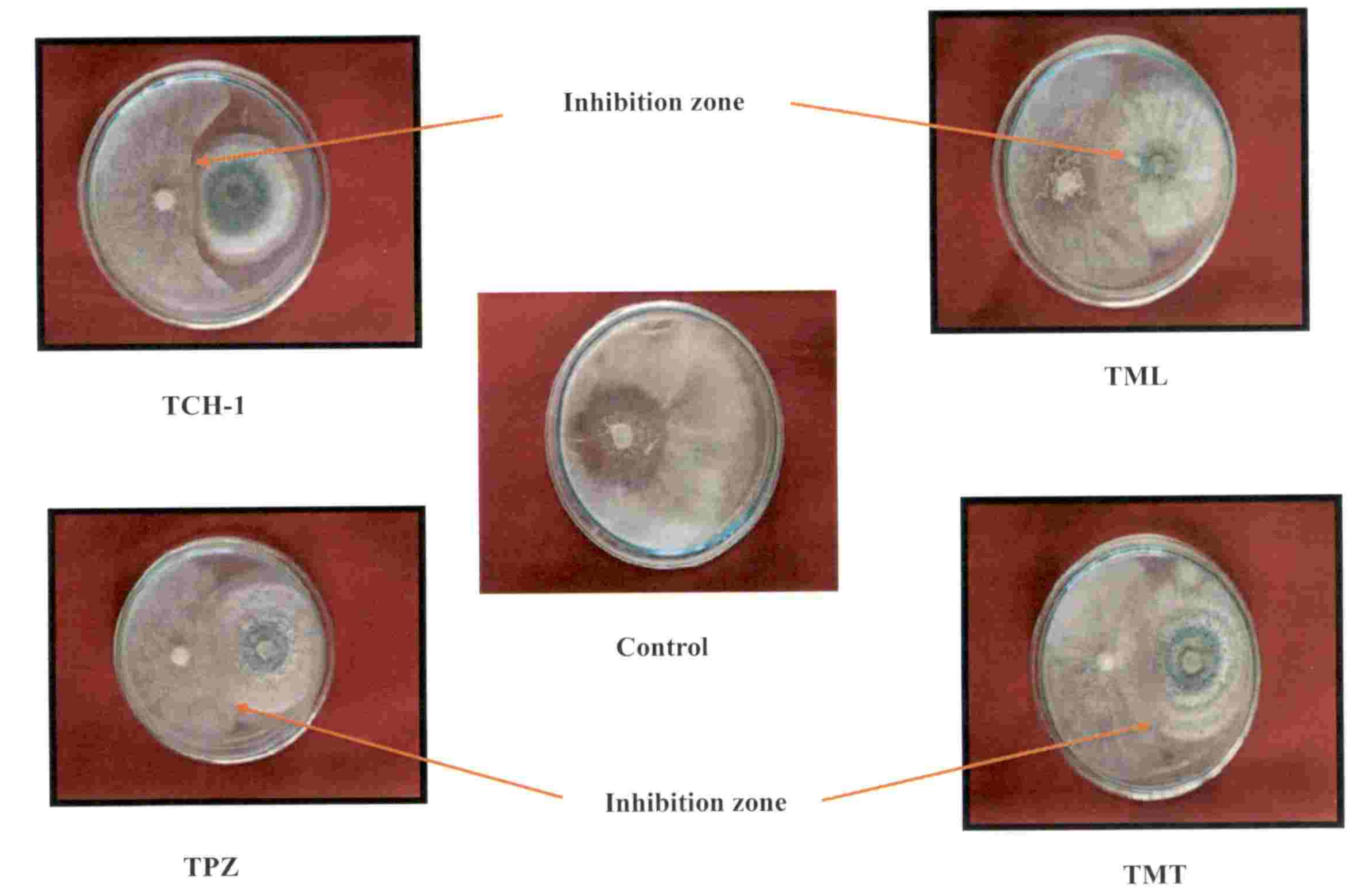


Plate 12. Antagonistic activity of *Trichoderma* sp. against *Pythium aphanidermatum*

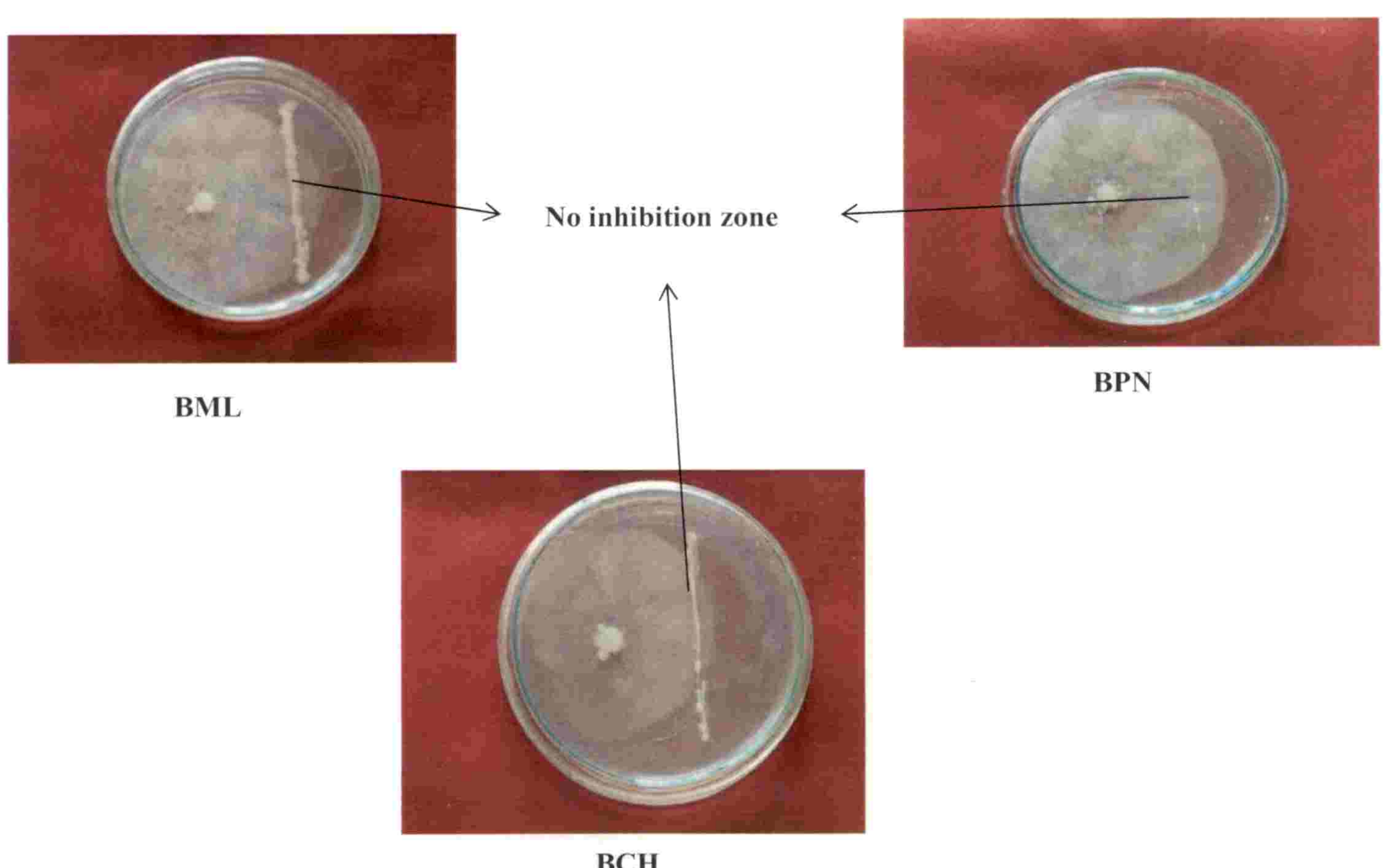
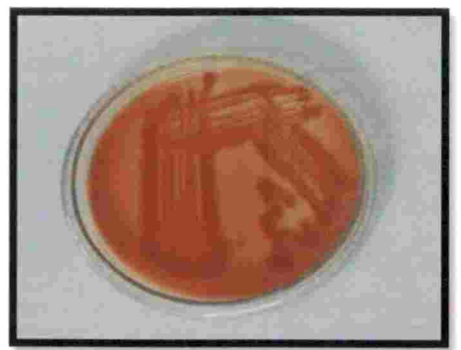
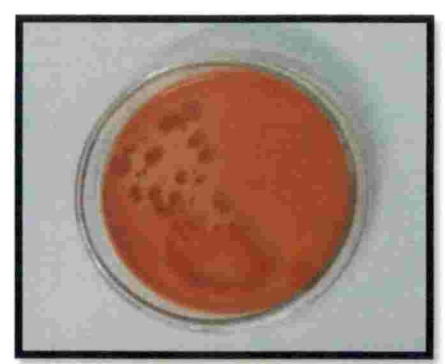


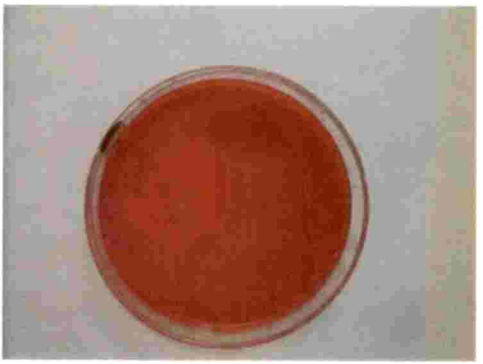
Plate 13. Antagonistic activities of *Bacillus* sp. against *Pythium aphanidermatum*



BCH (Biofilm +ve)

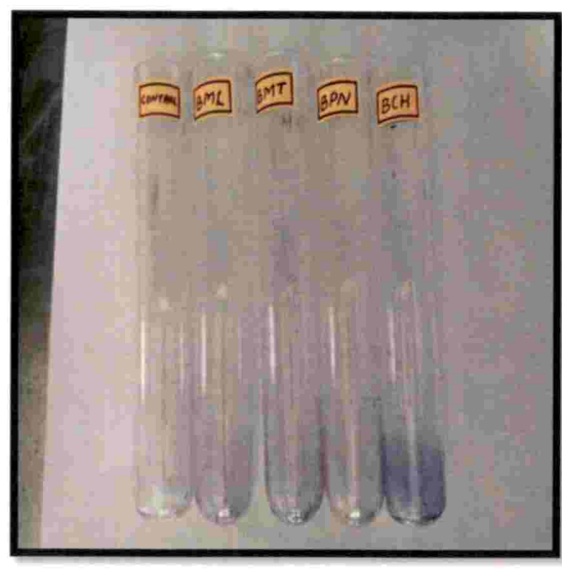


BMT (Biofilm +ve)



BMS (Biofilm –ve)

A. Congo red agar medium method



B. Tube method

C. Tissue culture plate method

Plate 14. Screening of *Bacillus* sp. for biofilm production

value (0.060) followed by BPN (0.056) isolate (Table 13). The least OD value (0.0556) was recorded in the case of BMT isolate.

Considering all the three methods, three most efficient biofilm producers (BCH, BPN, BMT) were selected for further studies.

4.7. Compatibility studies among the *Trichoderma* sp., *Bacillus* sp., and *Rhizobium* sp. under *in vitro*

4.7.1. Mutual compatibility between *Trichoderma* sp. and *Bacillus* sp. isolates under *in vitro*

The three promising *Bacillus* sp. (BCH, BPN, BML) and *Trichoderma* sp. (TCH, TMT, TPZ) isolates were tested for their compatibility with each other. No inhibition was observed at the intersection, indicating their compatibility to each other (Plate 15).

Trichoderma sp. (KAU) and *Bacillus* sp. (KAU) also showed no inhibition at the intersection, indicating their compatibility to each other (Plate 15).

4.7.2. Mutual compatibility between *Trichoderma* sp. and *Rhizobium* sp. under *in vitro*

The promising isolates of three *Trichoderma* sp. (TCH, TMT, TPZ) isolates were checked for their mutual compatibility with the *Rhizobium* sp. No inhibition was observed at the intersection, indicating their compatibility to each other (Plate 16).

Trichoderma sp. (KAU) and *Rhizobium* sp. did not show any inhibition, indicating their compatibility to each other.

4.7.3. Mutual compatibility between of *Bacillus* sp. and *Rhizobium* sp. under *in vitro*

Three selected *Bacillus* sp. (BCH, BPN, BML) were tested for their mutual compatibility with the *Rhizobium* sp. using cross streak method. No lysis was observed at the intersection, indicating their compatibility to each other (Plate 17).

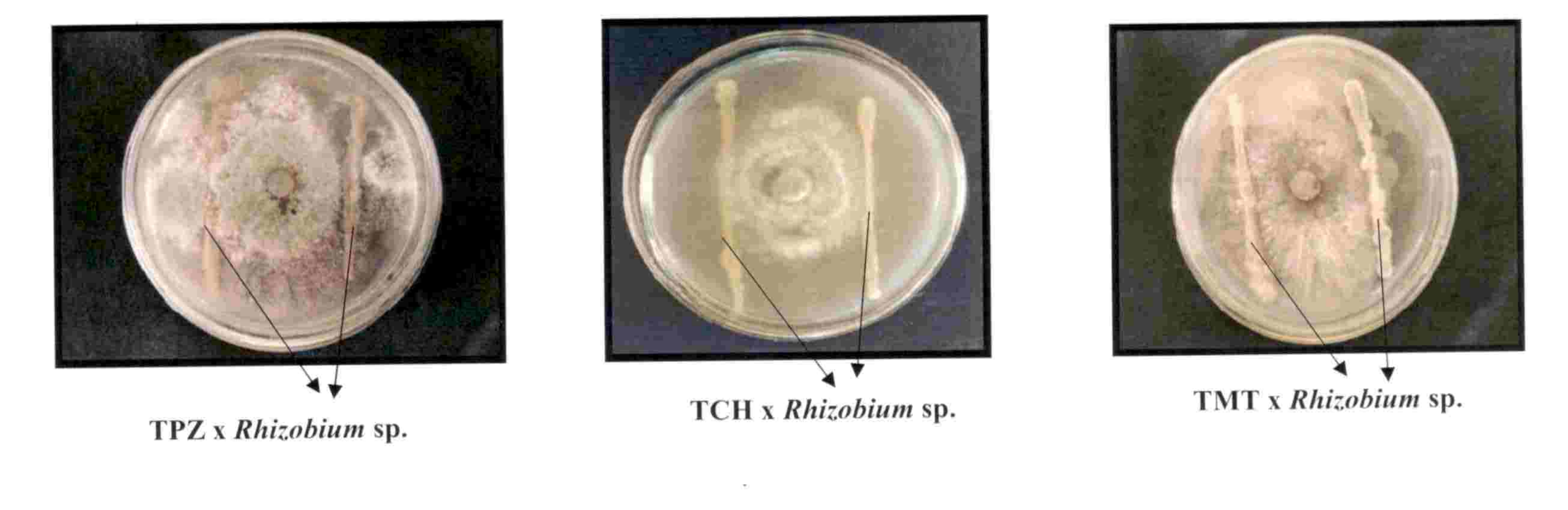
174329

Table 13. Screening of *Bacillus* sp. isolates for biofilm production under *in vitro*

Isolates	OD values (570 nm)
BCH (Chellakara)	0.060
BMS (Mullassery)	0.056
BML (Mala)	0.056
BPN (Pananchery)	0.058
BMT (Mattathur)	0.055

TCH-1　　　TMT　　　TPZ

Plate 15. Compatibility between *Trichoderma* sp. and *Bacillus* sp. under *in vitro*



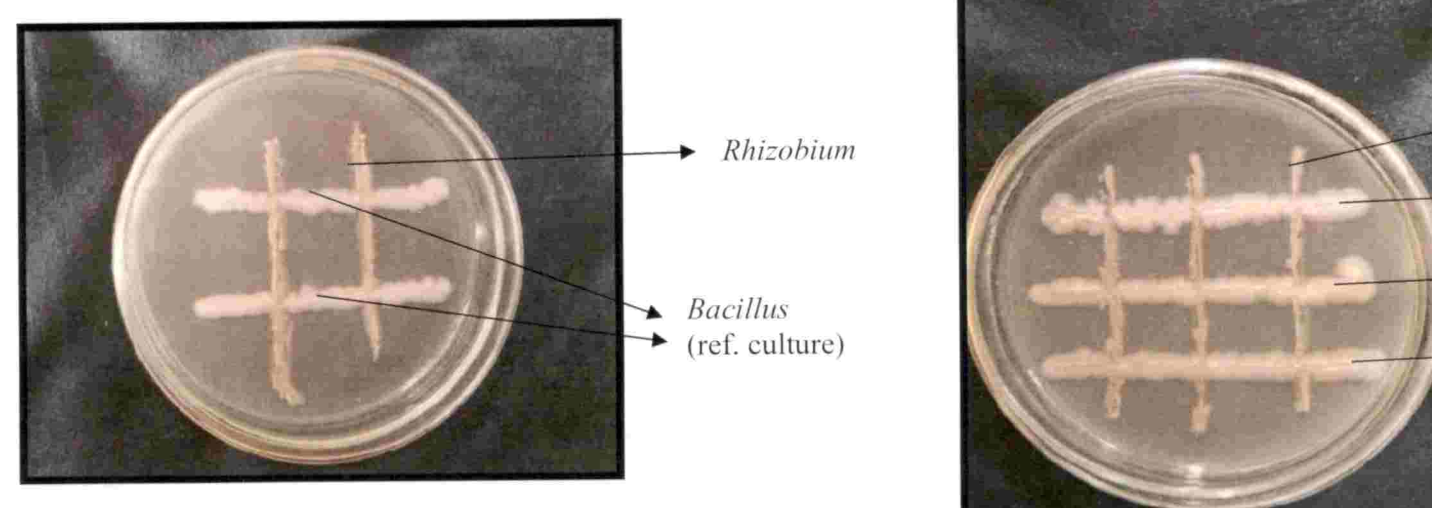
TPZ x *Rhizobium* sp.

TCH x *Rhizobium* sp.

TMT x *Rhizobium* sp.

Trichoderma sp. (KAU ref. culture) x *Rhizobium* sp.

Plate 17. Compatibility between different *Trichoderma* sp. and *Rhizobium sp.* under *in vitro*



Bacillus **sp. (KAU ref.culture) x *Rhizobium* sp.**

Bacillus **sp. x *Rhizobium* sp.**

Plate 18. Compatibility between different *Bacillus* sp. and *Rhizobium sp.*

Bacillus sp. (KAU) and *Rhizobium* sp. did not show lysis at the intersection, indicating their compatibility to each other.

4.8. Standardization of carrier material for *Trichoderma* based *Bacillus* sp. biofilm inoculant production

Standardization of carrier materials for *Trichoderma* based *Bacillus* sp. biofilm inoculant production were carried out using *viz.,* compost, vermiculite, talc powder, compost : vermiculite (1:1) and vermicompost as the carrier materials.

The population of *Trichoderma* sp. and *Bacillus* sp. were recorded at monthly interval (Table 14). At 30 DAI, *Trichoderma* sp. population was highest (97×10^5 cfu g^{-1}) in talc followed by vermiculite (62×10^5 cfu g^{-1}) and the lowest (53×10^5 cfu g^{-1}) population was recorded in vermicompost. At 60 DAI, the highest *Trichoderma* sp. (57×10^5 cfu g^{-1}) population was recorded in talc followed by vermiculite (39 x 10^5 cfu g^{-1}) and the lowest population (21×10^5 cfu g^{-1}) was recorded in compost. At 90 DAI, the population of *Trichoderma* sp. was highest (29×10^5 cfu g^{-1}) in talc followed by vermiculite (21×10^5 cfu g^{-1}). The lowest (14×10^5 cfu g^{-1}) was recorded in compost:vermiculite (1:1) which was on par with vermicompost.

Similarly, at 30 DAI the population of *Bacillus* sp. was highest (55×10^5 cfu g^{-1}) in vermicompost which was on par with talc and the lowest (38×10^5 cfu g^{-1}) population was recorded in compost. At 60 DAI, the population of *Bacillus* sp. was highest (28×10^5 cfu g^{-1}) in talc followed by compost. The lowest population of (15×10^5 cfu g^{-1}) was recorded in vermiculite. At 90 DAI, the population of *Bacillus* sp. was found to be highest (19×10^5 cfu g^{-1}) in talc followed by vermiculite (11×10^5 cfu g^{-1}). The lowest population (8×10^5 cfu g^{-1}) was recorded in vermicompost which was on par with compost: vermiculite. Based on the population at 90 DAI, the talc powder was found to be the most suitable carrier material.

4.9. Population of *Trichoderma* sp. and *Bacillus* sp. in biofilm based inoculum before and after mixing with talc powder

Population of *Trichoderma* sp. and *Bacillus* sp. in biofilm based media before and after mixing with the talc powder is presented in the Table 15. *Trichoderma* sp.

Table 14. Population of *Trichoderma* sp. and *Bacillus* sp. in biofilm based carrier material at monthly interval

Carrier material	Population (x 10^5 cfu g^{-1})					
	Trichoderma sp.			*Bacillus* sp.		
	30 DAI	60 DAI	90 DAI	30 DAI	60 DAI	90 DAI
Compost	55^c (6.74)	21^d (6.32)	16^c (6.20)	38^c (6.57)	18^b (6.25)	9^b (5.95)
Vermiculite	62^b (6.79)	39^b (6.59)	21^b (6.32)	41^c (6.12)	15^b (6.17)	11^b (6.04)
Talc powder	97^a (6.98)	57^a (6.75)	29^a (6.46)	53^a (6.72)	28^a (6.44)	19^a (6.28)
Compost : Vermiculite (1:1)	56^c (6.74)	31^c (6.49)	14^c (6.14)	46^b (96.62)	17^b (6.23)	8^b (5.90)
Vermicompost	53^c (6.72)	31^c (6.49)	14^c (6.14)	55^a (6.74)	17^b (6.23)	8^b (5.90)
CD (0.05)	4.81	6.47	3.63	4.45	4.81	5.27

DAI – Days after inoculation

Figures in the parenthesis indicate log transformed values

Each value represents mean of three replication

Table 15. Population of *Trichoderma* sp. and *Bacillus* sp. before and after mixing with the talc powder

Biofilm	Population			
	Before mixing with talc		After mixing with talc (48 h)	
	Trichoderma sp. (x 10^7 cfu ml^{-1})	*Bacillus* sp. (x 10^8 cfu ml^{-1})	*Trichoderma* sp. (x 10^7 cfu ml^{-1})	*Bacillus* sp. (x 10^8 cfu ml^{-1})
TCH + BCH	4.1^b (7.61)	3^b (4.47)	2.6^a (7.41)	2.6^a (8.41)
TMT + BML	4.4^b (7.64)	3.2^a (8.50)	2.4^b (7.38)	1.9^b (8.27)
TPZ + BPN	4.9^a (7.69)	2.9^b (8.46)	1.9^c (7.27)	2.1^b (8.32)

TCH : Chellakkara, TMT : Mattathur TPZ: Pazhayanur

BCH : Chellakkara, BML : Mala BPN : Pananchery

T: *Trichoderma* sp. B: *Bacillus* sp.

Each value represents mean of three replications NS – Non significant

Values in the parenthesis indicate log transformed values

population in the three most promising biofilm inoculum before mixing with talc powder were 4.1 x 10^7 cfu ml^{-1}, 4.4 x 10^7 cfu ml^{-1} and 4.9 x 10^7 cfu ml^{-1} in TCH+BCH Biofilm, TMT+BML biofilm and TPZ+BPN biofilm respectively. But after mixing with the talc powder, population of *Trichoderma* sp. in the biofilm inoculum were 2.6 × 10^7 cfu g^{-1}, 2.4 × 10^7 cfu g^{-1} and 1.9 × 10^7 cfu g^{-1} in TCH+BCH biofilm, TMT+BML biofilm and TPZ+BPN biofilm respectively.

Population of *Bacillus* sp. in the three most promising biofilm inoculum before mixing with talc powder were 3 x 10^8 cfu ml^{-1}, 3.2 x 10^8 cfu ml^{-1} and 2.9 x 10^8 cfu ml^{-1} in TCH+BCH biofilm, TMT+BML biofilm and TPZ+BPN biofilm respectively. But after mixing with the talc powder, population of *Bacillus* sp. in the biofilm inoculum were 2.6 × 10^7 cfu g^{-1}, 2.4 x 10^7 cfu g^{-1} and 1.9 × 10^7 cfu g^{-1} in TCH+BCH biofilm, TMT+BML biofilm and TPZ+BPN biofilm respectively.

4.10. Evaluation of biofilm based microbial antagonists for disease management and growth promotion in cowpea under pot culture studies

Three most promising *Trichoderma* based *Bacillus* sp. biofilm inoculants with talc as carrier material were evaluated for their efficiency in disease management and growth promotion in cowpea under pot culture.

4.11.1. Effect of different treatments on management of *Rhizoctonia solani* and growth promotion in cowpea under pot culture

4.11.1.1. Effect of different treatments on germination of cowpea before challenge inoculation with Rhizoctonia solani

Effect of different treatments on germination of cowpea are presented (Table 16). The number of days taken for germination ranged from 2-4 days. All the treatments showed cent per cent (100 %) germination at 4 DAS. However, at 2 DAS, highest germination per cent (42.8 %) was recorded in TCH+BCH biofilm based formulation (T_2) and in PGPR Mix – II (T_9). The lowest germination per cent (14.28 %) was recorded in *Bacillus* sp. (T_4) which was on par with Carbendazim +Mancozeb (T_7).

Table 16. Effect of different treatments on germination of cowpea before challenge inoculation with *Rhizoctonia solani* (30 DAS) under pot culture experiment

Treatments	Germination percent @ 2 DAS (%)	Germination percent @ 3 DAS (%)	Germination percent @ 4 DAS (%)
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	24.28	85.71	100
T_2 : Biofilm based *Trichoderma* sp.(TCH) + *Bacillus* sp. (BCH)	42.8	85.71	100
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	24.28	71.42	100
T_4 : *Bacillus* sp. (KAU ref.culture)	14.28	57.14	100
T_5 : *Trichoderma* sp. (KAU ref.culture)	24.28	71.42	100
T_6 : *Bacillus* sp. + *Trichoderma* sp.	24.28	57.14	100
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	14.28	42.8	100
T_8 : Package of practices (KAU, 2011)	24.28	57.14	100
T_9 : PGPR mix – II @ 2.5 kg / ha	42.8	87.71	100
T_{10} : Absolute control	24.28	71.42	100

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011) DAS : Days after sowing

T_9 : Organic Package (KAU, 2009) + PGPR mix – II @ 2.5 kg / ha

4.11.1.2. Effect of different treatments on plant height before and after the challenge inoculation with Rhizoctonia solani (30 DAS)

The cowpea plant height was recorded at fortnightly interval till the final harvest. The result showed increasing trend in plant height and showed significant differences among the treatments (Table 17). However, at final harvest, T_2 (TCH+BCH) recorded maximum plant height (124.14 cm) followed by T_9 (PGPR Mix- II) (117.14 cm) which was on par with T_6 (*Bacillus* sp.+*Trichoderma* sp.) (115.57 cm) and T_3 (TMT+BML) (112.57 cm). The lowest plant height (90.57 cm) was recorded in T_{10} (Control).

4.11.1.3. Effect of different treatments on number of leaves before and after the challenge inoculation of Rhizoctonia solani (30 DAS)

Number of leaves were recorded at fortnightly interval till the final harvest. The result showed increasing trend and recorded significant differences among the treatments (Table 18) However, at final harvest treatments were found to be non-significant.

In general, T_2 (TCH+BCH) and T_9 (PGPR Mix- II) recorded highest number of leaves from 15 DAS to 90 DAS.

At 15 DAS, T_1 (TPZ+BPN) recorded maximum number of leaves (9.28) which was on par with T_3 (TMT+BML) (9.28), T_2 (TCH+BCH) (8.85), T_5 (*Trichoderma* sp.) (8.42) and T_4 (*Bacillus* sp.) (8.42). The lowest (6.71) number of leaves was recorded in T_{10} (Control).

At 30 DAS, T_2 (TCH+BCH) recorded maximum number of leaves (28.85) followed by T_9 (PGPR Mix- II) (25.28). The lowest number of leaves (19.28) were recorded in T_{10} (Control).

At 45 DAS, maximum number of leaves (46.57) were observed in T_9 (PGPR Mix- II) which was on par with T_2 (TCH+BCH) (45.85). Least number of leaves (31.42) was recorded in T_{10} (Control).

Table 17. Effect of different treatments on plant height of cowpea before and after the challenge inoculation with *Rhizoctonia solani* (30 DAS) under pot culture experiment

Treatments	Plant height (cm)					
	Days after sowing					
	Before challenge inoculation		After challenge inoculation			
	15	30	45	60	75	90
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	35.90^{ab}	81.0	109.0^{b}	112.00^{b}	113.85^{abc}	112.28^{ab}
T_2 : Biofilm based *Trichoderma* sp.(TCH) + *Bacillus* sp. (BCH)	31.25^{e}	97.28	120.71^{a}	126.57^{a}	128.85^{a}	124.14^{a}
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	35.01^{abc}	77.71	107.85^{b}	114.42^{ab}	116.00^{abc}	112.57^{ab}
T_4 : *Bacillus* sp. (KAU ref.culture)	36.10^{ab}	76.85	109.28^{b}	110.85^{b}	112.85^{bc}	107.57^{bc}
T_5 : *Trichoderma* sp. (KAU ref.culture)	34.08^{bcd}	75.14	109.7^{ab}	114.42^{ab}	127.14^{ab}	108.85^{bc}
T_6 : *Bacillus* sp. + *Trichoderma* sp.	34.92^{abc}	78.57	111.57^{ab}	115.42^{ab}	116.42^{abc}	115.57^{ab}
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	32.47^{cde}	70.42	100.85^{bc}	103.14^{bc}	104.14^{cd}	98.57^{bc}
T_8 : Package of practices (KAU, 2011)	31.37^{de}	73.14	108.57^{b}	108.71^{b}	112.28^{bc}	109.14^{bc}
T_9 : PGPR mix – II @ 2.5 kg / ha	37.17^{a}	79.71	111.71^{ab}	114.71^{ab}	120.14^{ab}	117.14^{ab}
T_{10} : Absolute control	28.08^{f}	64.00	92.0^{c}	92.00^{c}	94.85^{d}	90.57^{c}
CD (5%)	2.804	NS	11.20	11.29	12.602	21.181

Each value represents mean of seven replications NS – Non significant DAS: Days after sowing

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR mix – II @ 2.5 kg / ha

Table 18. Effect of different treatments on number of leaves before and after the challenge inoculation with *Rhizoctonia solani* (30 DAS) under pot culture experiment

Treatments	Number of leaves					
	Days after sowing					
	Before challenge inoculation		After challenge inoculation			
	15	30	45	60	75	90
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	9.28^{a}	24.71^{abc}	41.42^{ab}	48.00^{ab}	49.14^{abc}	48.42
T_2 : Biofilm based *Trichoderma* sp.(TCH) + *Bacillus* sp. (BCH)	8.85^{a}	25.85^{a}	45.85^{a}	49.85^{a}	50.57^{a}	45.85
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	9.28^{a}	23.57^{bcd}	42.28^{ab}	48.42^{ab}	49.28^{abc}	38.28
T_4 : *Bacillus* sp. (KAU ref.culture)	8.42^{a}	23.57^{bcd}	43.57^{ab}	46.57^{ab}	49.85^{ab}	38.50
T_5 : *Trichoderma* sp. (KAU ref.culture)	8.42^{a}	23.42^{bcd}	37.28^{bc}	44.85^{ab}	48.85^{abc}	43.00
T_6 : *Bacillus* sp. + *Trichoderma* sp.	8.00^{ab}	23.00^{cd}	40.71^{ab}	41.14^{bc}	43.14^{bcd}	45.00
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	8.00^{ab}	23.71^{abc}	36.71^{bc}	41.71^{bc}	42.28^{cd}	43.57
T_8 : Package of practices (KAU, 2011)	8.00^{ab}	21.42^{de}	40.42^{ab}	42.57^{abc}	44.42^{abc}	34.14
T_9 : PGPR mix – II @ 2.5 kg / ha	8.85^{ab}	25.28^{ab}	46.57^{a}	48.71^{ab}	50.57^{a}	37.85
T_{10} : Absolute control	6.71^{b}	19.28^{e}	31.42^{c}	35.42^{c}	36.57^{d}	27.14
CD (5 %)	1.31	2.27	7.71	7.97	7.06	NS

Each value represents mean of seven replications NS – Non significant

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR mix – II @ 2.5 kg / ha

At 60 DAS, maximum number of leaves (49.85) were observed in T_2 (TCH+BCH) followed by T_9 (PGPR Mix- II) (48.71) which was on par with all other treatments except T_8 (POP KAU, 2011) (42.57), T_7 (Carbendazim+Mancozeb) (41.71) and T_6 (*Bacillus* sp.+ *Trichoderma* sp.) (41.14). The minimum number of leaves (35.42) was recorded in T_{10} (Control).

At 75 DAS, maximum number of leaves (50.57) were recorded in T_2 (TCH+BCH) which was on par with T_9 (PGPR Mix- II) (5.57). The minimum number of leaves (36.57) was recorded in T_{10} (Control).

At 90 DAS, there were no significant differences among the treatments.

4.11.1.4. Effect of different treatments on the number taken for first flowering in cowpea with challenge inoculation with Rhizoctonia solani (30 DAS)

The number of days taken for first flowering ranged between 54-59 days. The treatments did not show any significant differences among the treatments (Table 19). However, the minimum days (54) taken for first flowering was recorded in T_3 (TMT+BML) followed by T_2 (TCH+BCH) (54.28) and T_9 (PGPR Mix- II) (54.28). The maximum number of days (59) taken for first flowering was in the case of T_5 (*Trichoderma* sp.).

4.11.1.5. Effect of different treatments on fresh and dry weight of cowpea after challenge inoculation with Rhizoctonia solani (30 DAS)

There were no significant differences among the treatments with respect to fresh weight (Table 20). However, the maximum fresh weight (71.50 g plant^{-1}) was recorded in T_9 (PGPR Mix- II) followed by T_2 (TCH+BCH) (70.37 g plant^{-1}). The lowest fresh weight (38.50 g/plant) was recorded in T_{10} (Control).

Similarly, there were no significant differences among the treatments with respect to dry weight (Table 20). However, the maximum dry weight (11.25 g plant^{-1}) was observed in T_2 (TCH+BCH) followed by T_4 (*Bacillus* sp.) (10 g plant^{-1}), T_7 (Carbendazim +Mancozeb) and T_1 (TPZ+BPN). The minimum dry weight (7 g plant^{-1}) was recorded in T_{10} (Control).

Table 19. Effect of different treatments on the number of days taken for first flowering in cowpea after challenge inoculation with *Rhizoctonia solani* (30 DAS)

Treatments	Days taken for first flowering
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	56.0
T_2 : Biofilm based *Trichoderma* sp. (TCH) + *Bacillus* sp. (BCH)	54.28
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	54.0
T_4 : *Bacillus* sp. (KAU ref.culture)	56.14
T_5 : *Trichoderma* sp. (KAU ref.culture)	59.0
T_6 : *Bacillus* sp. + *Trichoderma* sp.	55.57
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	58.57
T_8 : Package of practices (KAU, 2011)	55.85
T_9 : PGPR Mix – II @ 2.5 kg / ha	54.28
T_{10} : Absolute control	58.57
CD (5 %)	NS

Each value represents mean of seven replications

NS – non significant

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR Mix – II @ 2.5 kg / ha

Table 20. Effect of different treatments on fresh and dry weight of cowpea after challenge inoculation with *Rhizoctonia solani* (30 DAS) under pot culture experiment

Treatments	Fresh weight 100 DAS (g $plant^{-1}$)	Dry weight 110 DAS (g $plant^{-1}$)
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	68.16	10.0
T_2 : Biofilm based *Trichoderma* sp. (TCH) + *Bacillus* sp. (BCH)	70.37	11.25
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	66.0	7.33
T_4 : *Bacillus* sp. (KAU ref.culture)	49.75	10.0
T_5 : *Trichoderma* sp. (KAU ref.culture)	62.50	7.83
T_6 : *Bacillus* sp. + *Trichoderma* sp.	53.33	7.40
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	51.0	10.0
T_8 : Package of practices (KAU, 2011)	45.25	8.66
T_9 : PGPR Mix – II @ 2.5 kg / ha	71.50	9.60
T_{10} : Absolute control	38.50	7.0
CD (5 %)	NS	NS

Each value represents mean of seven replications NS – non significant T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR Mix – II @ 2.5 kg / ha

4.11.1.6. Effect of different treatments on yield after challenge inoculation with Rhizoctonia solani (30 DAS)

The treatments did not show any significant differences (Table 21). However, T_9 (PGPR Mix- II) recorded highest yield (52.28 g plant^{-1}) followed by T_6 (*Bacillus* sp. +*Trichoderma* sp.) with 48.49 g plant^{-1} which was on par with T_2 (TCH+BCH). The lowest yield of 39.85 g plant^{-1} was recorded in T_{10} (Control).

4.11.1.7. Effect of different treatments on per cent collar rot disease incidence caused by Rhizoctonia solani after challenge inoculation (30 DAS)

After the artificial inoculation of *Rhizoctonia solani* (Plate 18), per cent collar rot disease were recorded at fortnightly interval (Table 22).

At 40 DAS, only T_2 (TCH+BCH) and T_9 (PGPR Mix- II) did not show any disease incidence whereas the highest per cent disease incidence (57.14 %) was observed in T_{10} (Control). Even at 55 DAS and 70 DAS, the per cent disease incidence remained same at final harvest in the case of T_2 (TCH+BCH) and T_9 (PGPR Mix- II). However, at 85 DAS, T_2 (TCH+BCH), T_5 (*Trichoderma* sp.), T_7 (Carbendazim +Mancozeb) and T_9 (PGPR Mix- II) recorded minimum (14.28 %) per cent disease incidence. The highest per cent (57.14 %) disease incidence was recorded in the case of T_{10} (Control).

On the whole, T_2 and T_9 (Treatments) recorded lowest disease incidence.

4.11.1.8. Effect of different treatments on nutrient status of the potting mixture at harvest after challenge inoculation with Rhizoctonia solani (30 DAS)

4.11.1.8.1. pH

There were significant differences in the pH of the potting mixture (Table 23). The initial pH of the potting mixture was 7.09. At final harvest, the highest pH (7.1) was observed in the case of T_2 (TCH+BCH) and the lowest pH (6.59) was in the case of T_9 (PGPR Mix- II).

Plate 18. Artificial inoculation of *Rhizoctonia solani* on cowpea

T_2 : Biofilm based TCH+BCH

T_9 : PGPR Mix – II

T_5 : *Trichoderma* sp.

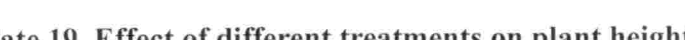


Plate 19. Effect of different treatments on plant height

Table 21. Effect of different treatments on yield of cowpea after challenge inoculation with *Rhizoctonia solani* (30 DAS) under pot culture experiment

Treatments	Yield (g plant^{-1}) After 100 DAS	Projected yield (t ha^{-1})
T_1 : Biofilm based *Trichoderma* sp. (TPZ) +*Bacillus* sp. (BPN)	45.42^{bcd}	3.36
T_2 : Biofilm based *Trichoderma* sp. (TCH) + *Bacillus* sp. (BCH)	48.00^{bc}	3.55
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	44.85^{cd}	3.32
T_4 : *Bacillus* sp. (KAU ref.culture)	43.42^{d}	3.21
T_5 : *Trichoderma* sp. (KAU ref.culture)	44.85^{cd}	3.32
T_6 : *Bacillus* sp. + *Trichoderma* sp.	48.49^{b}	3.59
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	45.14^{bcd}	3.34
T_8 : Package of practices (KAU, 2011)	44.57^{d}	3.30
T_9 : PGPR Mix – II @ 2.5 kg / ha	52.28^{a}	3.87
T_{10} : Absolute control	39.85^{e}	2.95
CD (5 %)	3.31	-

Each value represents mean of seven replications

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR Mix – II @ 2.5 kg / ha

Table 22. Effect of different treatments on per cent collar rot disease caused by *Rhizoctonia solani* after challenge inoculation (30 DAS) under pot culture experiment

Treatments	Per cent disease incidence (40 DAS)	Per cent disease incidence (55 DAS)	Per cent disease incidence (70 DAS)	Per cent disease incidence (85 DAS)
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	14.28	14.28	14.28	28.57
T_2 : Biofilm based *Trichoderma* sp. (TCH) + *Bacillus* sp. (BCH)	0	0	0	14.28
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	14.28	14.28	28.57	28.57
T_4 : *Bacillus* sp. (KAU ref.culture)	28.57	28.57	28.57	28.57
T_5 : *Trichoderma* sp. (KAU ref.culture)	14.28	14.28	14.28	14.28
T_6 : *Bacillus* sp. + *Trichoderma* sp.	42.85	42.85	42.85	42.85
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	14.28	14.28	14.28	14.28
T_8 : Package of practices (KAU, 2011)	42.85	42.85	42.85	42.85
T_9 : PGPR Mix – II @ 2.5 kg / ha	0	0	0	14.28
T_{10} : Absolute control	57.14	57.14	57.14	57.14

Each value represents mean of seven replications

DAS : Days after sowing

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR Mix – II @ 2.5 kg / ha

Table 23. Effect of different treatments on the nutrient status of the potting mixture at harvest after challenge inoculation with *Rhizoctonia solani* (30 DAS)

Treatments	pH	EC (dSm^{-1})	Organic carbon (%)	Available N ($mg\ kg^{-1}$)	Available P ($mg\ kg^{-1}$)	Available K ($mg\ kg^{-1}$)
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	6.88^{bc}	2.68^{b}	2.06^{c}	112.00^{c}	635.0^{d}	521.5^{f}
T_2 : Biofilm based *Trichoderma* sp.(TCH) + *Bacillus* sp. (BCH)	6.99^{ab}	2.47^{c}	1.76^{g}	140.00^{b}	710.0^{b}	578.0^{c}
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	7.10^{a}	2.74^{b}	1.95^{d}	139.95^{b}	775.0^{a}	586.0^{b}
T_4 : *Bacillus* sp. (KAU ref.culture)	6.90^{ab}	3.30^{a}	2.17^{b}	168.00^{a}	605.0^{e}	547.0^{d}
T_5 : *Trichoderma* sp. (KAU ref.culture)	6.99^{c}	2.69^{b}	2.51^{a}	168.00^{a}	710.0^{b}	525.5^{ef}
T_6 : *Bacillus* sp. + *Trichoderma* sp.	6.74^{ab}	2.47^{c}	2.15^{b}	168.00^{a}	510.0^{g}	483.5^{g}
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	6.94^{ab}	2.47^{c}	1.86^{e}	139.95^{b}	467.5^{h}	403.0^{i}
T_8 : Package of practices (KAU, 2011)	6.94^{ab}	2.01^{d}	1.81^{f}	139.95^{b}	530.0^{f}	530.0^{e}
T_9 : PGPR mix – II @ 2.5 kg / ha	6.59^{bc}	2.12^{d}	1.77^{g}	168.00^{b}	600.0^{e}	461.5^{h}
T_{10} : Absolute control	6.88^{d}	2.76^{b}	1.97^{d}	139.95^{b}	680.0^{c}	634.5^{a}
CD (5 %)	0.16	0.11	0.03	7.66	7.34	7.06

Each value represents mean of three replications

Initial pH =7.09 Initial organic carbon =3.05% Electrical conductivity = 0.47 dSm^{-1}

Available nitrogen = 373.74 $mg\ kg^{-1}$ Available phosphorus = 72.70 $mg\ kg^{-1}$ Available potassium =917.09 $mg\ kg^{-1}$

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR mix – II @ 2.5 kg / ha

100

4.11.1.8.2. Electrical conductivity

Initial electrical conductivity of the potting mixture was 0.47 dS m^{-1} (Table 23). The maximum electrical conductivity (3.3 dS m^{-1}) was recorded in T_4 (*Bacillus* sp.) and the lowest electrical conductivity (2.01 dSm^{-1}) was recorded in T_8 (POP KAU, 2011) at harvest.

4.11.1.8.3. Organic carbon content

The different treatments resulted in significant differences with respect to organic carbon percentage (Table 23). Initial organic carbon content of the potting mixture was 3.05 per cent. At final harvest, T_5 (*Trichoderma* sp.) recorded maximum organic carbon content (2.51 %) and the lowest (1.76 %) content was recorded in T_2 (TCH+BCH).

4.11.1.8.4. Available nitrogen

The initial available nitrogen content was 373.74 mg kg^{-1} and decreased at the final harvest (Table 23). The maximum available nitrogen (168 mg kg^{-1}) was recorded in T_4 (*Bacillus* sp.) which was on par with T_5 (*Trichoderma* sp.), T_6 (*Bacillus* sp.+*Trichoderma* sp.) and T_7 (Carbendazim + Mancozeb). The lowest available N content was recorded in T_1 (TPZ+BPN) with 112 mg kg^{-1}.

4.11.1.8.5. Available phosphorus

The data revealed that there were significant differences among the treatments (Table 23). The initial available phosphorus content of the potting mixture was 72.70 mg kg^{-1}. At final harvest, highest available P content of the potting mixture was recorded in T_3 (BML + TMT) with 775 mg kg^{-1} and the lowest available P content was recorded in T_7 (Carbendazim +Mancozeb) with 467.5 mg kg^{-1}.

4.11.1.8.6. Available potassium

Analysis of the data revealed significant differences among the treatments (Table 23). The initial available potassium content of the potting mixture was 917.09 mg kg^{-1}. At final harvest, maximum available K content was recorded in T_9 (PGPR

Mix- II) with 634.5 mg kg^{-1} and the minimum available K content was recorded in T_7 (Carbendazim +Mancozeb) with 403 mg kg^{-1}.

4.11.1. Effect of different treatments on management of *Pythium aphanidermatum* and growth promotion in cowpea under pot culture

4.12.1.1. Effect of different treatments on the germination of cowpea before challenge inoculation with Pythium aphanidermatum

The number of days taken for germination ranged from 2-4 days (Table 24). All the treatments showed cent per cent (100 %) germination at 4 DAS. However, at 2 DAS, highest germination per cent (42.8 %) was recorded in T_2 (TCH+BCH) and T_9 (PGPR Mix – II). The lowest germination per cent (14.28 %) was recorded in T_4 (*Bacillus* sp.), T_3 (TMT+BML) and T_6 (*Bacillus* sp.+ *Trichoderma* sp.).

4.12.1.2. Effect of different treatments on plant height before and after challenge inoculation with Pythium aphanidermatum (30 DAS)

Plant height of cowpea was recorded at fortnightly interval till the harvest. The result showed increasing trend in plant height and there were significant differences among the treatments (Table 25). However, at final harvest, the highest plant height was recorded in T_5 (*Trichoderma* sp.) with 123.85 cm followed by T_9 (PGPR Mix- II) with 120.1 cm. The lowest plant height was recorded in T_{10} (Control) with 96.85 cm.

4.12.1.3. Effect of different treatments on number of leaves before and after challenge inoculation with Pythium aphanidermatum (30 DAS)

Number of leaves were recorded at fortnightly interval till the final harvest. The results showed significant differences among the treatments (Table 26). However, at final harvest, the maximum number of leaves were recorded in T_7 (Carbendazim + Mancozeb) with 61.75 leaves followed by T_1 (TPZ+BPN) (46.71) which was on par with T_5 (*Trichoderma* sp.) (45.60).

Table 24. Effect of different treatments on the germination of cowpea before challenge inoculation of *Pythium aphanidermatum* (30 DAS) under pot culture experiment

Treatments	Germination per cent @ 2 DAS	Germination per cent @ 3 DAS	Germination per cent @ 4 DAS
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	42.85	85.71	100
T_2 : Biofilm based *Trichoderma* sp.(TCH) + *Bacillus* sp. (BCH)	24.28	71.42	100
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	14.28	57.14	100
T_4 : *Bacillus* sp. (KAU ref.culture)	14.28	42.8	100
T_5 : *Trichoderma* sp. (KAU ref.culture)	24.28	57.14	100
T_6 : *Bacillus* sp. + *Trichoderma* sp.	14.28	42.8	100
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	42.28	85.71	100
T_8 : Package of practices (KAU, 2011)	24.28	71.42	100
T_9 : PGPR Mix – II @ 2.5 kg / ha	42.85	71.42	100
T_{10} : Absolute control	24.28	57.14	100

DAS : Days after sowing

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR Mix – II @ 2.5 kg / ha

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Table 25. Effect of different treatments on plant height of cowpea before and after the challenge inoculation with *Pythium aphanidermatum* (30 DAS) under pot culture experiment

Treatments	Plant height (cm)					
	Days after sowing					
	Before challenge inoculation		After challenge inoculation			
	15 DAS	30 DAS	45 DAS	60 DAS	75 DAS	90 DAS
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	24.04^{a}	91.14^{ab}	108.14	110.42	112.42^{a}	115.42^{ab}
T_2 : Biofilm based *Trichoderma* sp.(TCH) + *Bacillus* sp. (BCH)	26.31^{a}	97.57^{a}	116.14	117.57	118.28^{a}	118.57^{ab}
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	25.04^{a}	94.90^{a}	106.14	113.00	113.14^{a}	116.0^{ab}
T_4 : *Bacillus* sp. (KAU ref.culture)	24.35^{a}	88.28^{ab}	107.71	113.14	114.57^{a}	114.57^{ab}
T_5 : *Trichoderma* sp. (KAU ref.culture)	25.60^{a}	76.71^{cd}	109.00	112.71	119.14^{a}	123.85^{a}
T_6 : *Bacillus* sp. + *Trichoderma* sp.	25.95^{a}	76.71^{cd}	89.57	110.14	116.00^{a}	116.42^{ab}
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	25.60^{a}	88.71^{ab}	110.14	111.28	113.28^{a}	108.14^{bc}
T_8 : Package of practices (KAU, 2011)	24.24^{a}	81.57^{bc}	101.28	110.14	111.14^{a}	112.2^{ab}
T_9 : PGPR Mix – II @ 2.5 kg / ha	26.25^{a}	96.57^{a}	113.28	118.25	119.85^{a}	120.1^{ab}
T_{10} : Absolute control	20.12^{b}	67.42^{d}	87.85	96.14	95.28^{b}	94.85^{c}
CD (5 %)	2.920	10.46	NS	NS	13.29	13.57

Each value represents mean of seven replications NS – Non significant

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR Mix – II @ 2.5 kg / ha

Table 26. Effect of different treatments on number of leaves before and after the challenge inoculation of *Pythium aphanidermatum* (30 DAS) under pot culture experiment

Treatments	Number of leaves					
	Days after sowing					
	Before challenge inoculation		After challenge inoculation			
	15 DAS	30 DAS	45 DAS	60 DAS	75 DAS	90 DAS
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	8.85	23.57^{ab}	42.42^{ab}	51.42^{ab}	52.57^{ab}	46.71^{b}
T_2 : Biofilm based *Trichoderma* sp.(TCH) + *Bacillus* sp. (BCH)	9.28	25.28^{a}	43.71^{ab}	57.28^{a}	57.00^{a}	40.75^{bc}
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	8.57	23.85^{ab}	42.00^{ab}	50.14^{b}	50.14^{b}	41.0^{bc}
T_4 : *Bacillus* sp. (KAU ref.culture)	9.28	22.42^{bc}	38.42^{ab}	51.14^{ab}	50.71^{ab}	35.00^{bc}
T_5 : *Trichoderma* sp. (KAU ref.culture)	8.42	24.00^{ab}	36.42^{bc}	48.00^{b}	50.00^{b}	45.60^{b}
T_6 : *Bacillus* sp. + *Trichoderma* sp.	8.00	21.28^{cd}	36.71^{bc}	50.14^{b}	50.42^{ab}	44.80^{bc}
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	8.00	23.85^{ab}	39.00^{ab}	49.42^{b}	52.57^{ab}	61.75^{a}
T_8 : Package of practices (KAU, 2011)	8.00	22.71^{bc}	35.85^{bc}	48.00^{b}	48.14^{b}	42.16^{bc}
T_9 : PGPR Mix – II @ 2.5 kg / ha	8.00	25.71^{a}	46.00^{a}	54.00^{ab}	53.71^{ab}	39.60^{bc}
T_{10} : Absolute control	8.00	19.57^{d}	29.71^{c}	39.42^{c}	40.71^{c}	32.20^{c}
CD (5 %)	NS	2.236	8.228	7.133	12.49	13.36

Each value represents mean of seven replications NS – Non significant

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR Mix – II @ 2.5 kg / ha

4.12.1.4. Effect of different treatments on the number of days taken for first flowering in cowpea after challenge inoculation with Pythium aphanidermatum (30 DAS)

The number of days taken for first flowering ranged from 52-58 days. There were no significant differences among treatments (Table 27). However, the minimum days (52.71) taken for first flowering was recorded in both T_2 (TCH+BCH) and T_3 (TMT+BML). The maximum number of days (58.42) taken for first flowering was recorded in T_5 (*Trichoderma* sp.).

4.12.1.5. Effect of different treatments on fresh and dry weight of cowpea after challenge inoculation with Pythium aphanidermatum (30 DAS)

There were no significant differences among the treatments with respect to fresh weight (Table 28). However, the maximum fresh weight (72.20 g plant^{-1}) was recorded in T_5 (*Trichoderma* sp.) followed by T_9 (PGPR Mix- II) (70.57 g plant^{-1}). The lowest fresh weight (49.71 g plant^{-1}) was recorded in T_{10} (Control).

4.12.1.6. Effect of different treatments on yield after the challenge inoculation with Pythium aphanidermatum (30 DAS)

There were no significant differences among the treatments (Table 29). However, the highest yield (51.71 g plant^{-1}) was recorded in T_9 (PGPR Mix- II) followed by T_6 (*Bacillus* sp.+*Trichoderma* sp.) with 49 g plant^{-1}. The lowest yield was recorded in T_{10} (Control) with 39.71 g plant^{-1}.

4.12.1.7. Effect of different treatments on per cent disease incidence after the challenge inoculation with Pythium aphanidermatum

After the artificial inoculation with *Pythium aphanidermatum*, per cent disease incidence was recorded at fortnightly interval. No disease incidence was observed throughout the experimental period.

Table 27. Effect of different treatments on number of days taken for first flowering in cowpea after challenge inoculation with *Pythium aphanidermatum* (30 DAS) under pot culture experiment

Treatments	Days taken for first flowering
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	56.00
T_2 : Biofilm based *Trichoderma* sp.(TCH) + *Bacillus* sp. (BCH)	52.71
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	52.71
T_4 : *Bacillus* sp. (KAU ref.culture)	55.85
T_5 : *Trichoderma* sp. (KAU ref.culture)	58.42
T_6 : *Bacillus* sp. + *Trichoderma* sp.	57.28
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	55.57
T_8 : Package of practices (KAU, 2011)	55.28
T_9 : PGPR Mix – II @ 2.5 kg / ha	55.28
T_{10} : Absolute control	50.85
CD (5 %)	NS

Each value represents mean of seven replications NS – Non significant

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR Mix – II @ 2.5 kg / ha

Table 28. Effect of different treatments on fresh and dry weight of cowpea after challenge inoculation with *Pythium aphanidermatum* (30 DAS) under pot culture experiment

Treatments	Fresh weight after 100 DAS (g $plant^{-1}$)	Dry weight After 110 DAS (g $plant^{-1}$)
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	51.00	6.8
T_2 : Biofilm based *Trichoderma* sp.(TCH) + *Bacillus* sp. (BCH)	63.71	10.50
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	54.833	8.40
T_4 : *Bacillus* sp. (KAU ref.culture)	45.20	8.5
T_5 : *Trichoderma* sp. (KAU ref.culture)	72.20	8.2
T_6 : *Bacillus* sp. + *Trichoderma* sp.	41.600	8.0
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	53.25	6.75
T_8 : Package of practices (KAU, 2011)	54.00	6.75
T_9 : PGPR Mix – II @ 2.5 kg / ha	70.57	9.25
T_{10} : Absolute control	49.71	7.75
CD (5 %)	NS	NS

NS – Not significant

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR Mix – II @ 2.5 kg / ha

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Table 29. Effect of different treatments on the yield of cowpea after challenge inoculation with *Pythium aphanidermatum* (30 DAS) under pot culture experiment

Treatments	Yield After 100 DAS (g $plant^{-1}$)	Projected yield (t ha^{-1})
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	47.00^{bc}	3.48
T_2 : Biofilm based *Trichoderma* sp.(TCH) + *Bacillus* sp. (BCH)	47.42^{bc}	3.51
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	45.28^{c}	3.35
T_4 : *Bacillus* sp. (KAU ref.culture)	44.28^{c}	3.27
T_5 : *Trichoderma* sp. (KAU ref.culture)	45.85^{bc}	3.39
T_6 : *Bacillus* sp. + *Trichoderma* sp.	49.00^{ab}	3.69
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	44.85^{c}	3.32
T_8 : Package of practices (KAU, 2011)	44.14^{c}	3.26
T_9 : PGPR Mix – II @ 2.5 kg / ha	51.71^{a}	3.83
T_{10} : Absolute control	39.71^{d}	2.94
CD (5 %)	3.63	-

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR Mix – II @ 2.5 kg / ha

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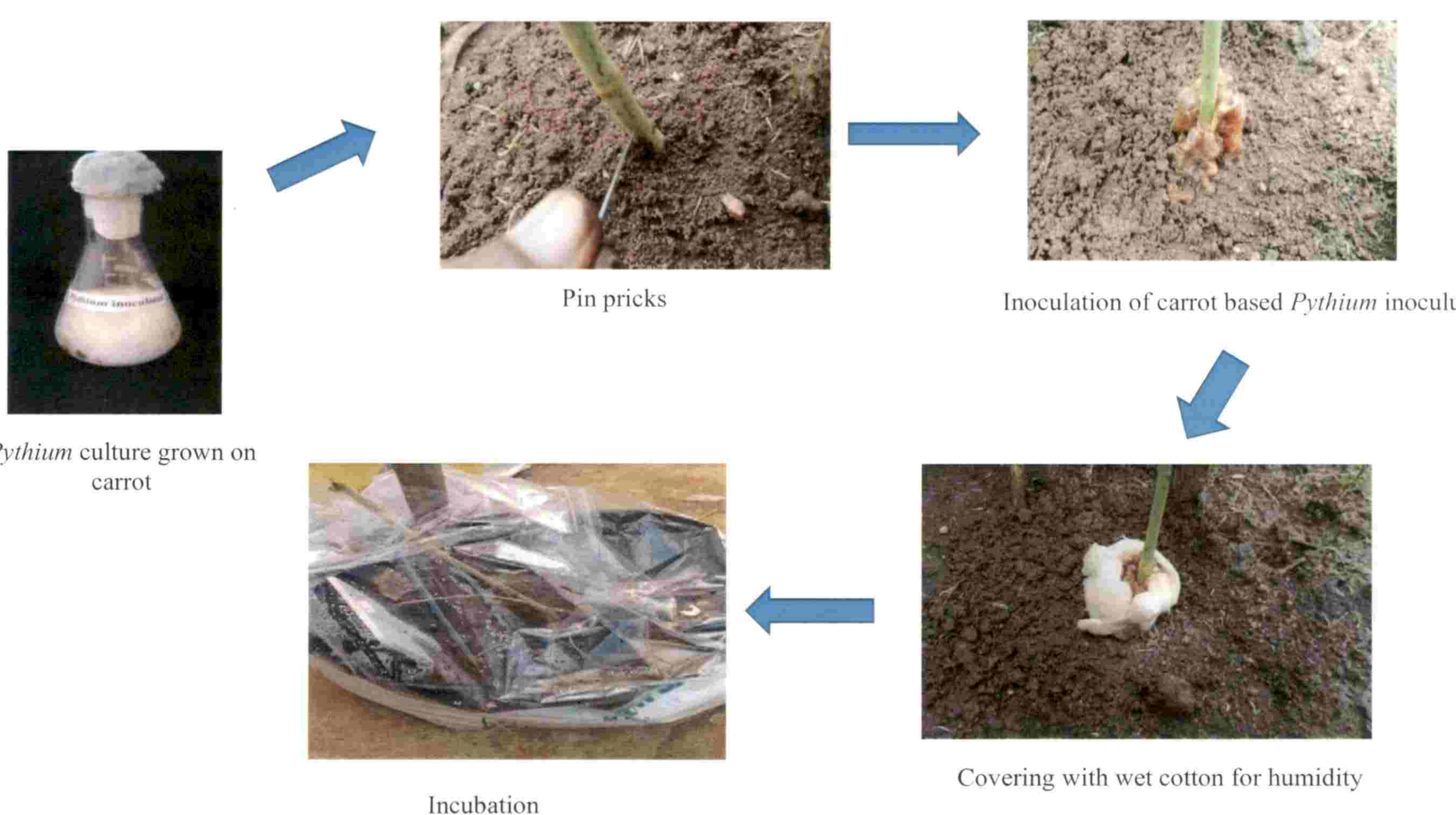


Plate 20. Artificial inoculation of *Pythium aphanidermatum* on cowpea

4.12.1.8. Effect of different treatments on nutrient status of potting mixture at harvest after challenge inoculation with Pythium aphanidermatum (30 DAS)

4.11.1.8.1. pH

There were significant differences among the treatments (Table 30). The initial pH of the potting mixture was 7.09. At harvest, the highest pH (9.4) was observed in the case of T_7 (Carbendazim + Mancozeb). The lowest pH (6.67) was in the case of T_5 (*Trichoderma* sp.).

4.12.1.8.2. Electrical conductivity

The initial electrical conductivity of the potting mixture was 0.47 dS m^{-1} (Table 30). The maximum electrical conductivity (3.3 dS m^{-1}) was recorded in T_4 (*Bacillus* sp.) and the lowest electrical conductivity (2.48 dS m^{-1}) was recorded in T_2 (TCH+BCH) at harvest.

4.12.1.8.3. Organic carbon content

The application of different treatments resulted in significant differences in the organic carbon percentage (Table 30). The initial organic carbon content of the potting mixture was 3.05 per cent. At harvest, T_9 (PGPR Mix- II) recorded maximum organic carbon content (2.47 %) and lowest content (1.56 %) was recorded in T_7 (Carbendazim + Mancozeb).

4.12.1.8.4. Available nitrogen

The initial available nitrogen content was 373.74 mg kg^{-1}, which decreased after the experiment (Table 30). The maximum available nitrogen (224 mg kg^{-1}) was recorded in T_6 (*Bacillus* sp.+*Trichoderma* sp.) which was on par with T_8 (POP KAU, 2011) (168 mg kg^{-1}). The lowest available nitrogen (112 mg kg^{-1}) was recorded in both T_3 (TMT+BML) and T_4 (*Bacillus* sp.).

4.12.1.8.5. Available phosphorus

The data revealed significant differences among treatment (Table 30). The initial available phosphorus content of the potting mixture was 72.70 mg kg^{-1}. At

Table 30. Effect of different treatments on nutrient status at harvest after challenge inoculation with *Pythium aphanidermatum* (30 DAS) under pot culture experiment

Treatments	pH	EC (dSm^{-1})	Organic carbon (%)	Available N ($mg\ kg^{-1}$)	Available P ($mg\ kg^{-1}$)	Available K ($mg\ kg^{-1}$)
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	6.81^c	2.67^e	2.04^c	168^c	620^d	428.5^c
T_2 : Biofilm based *Trichoderma* sp.(TCH) + *Bacillus* sp. (BCH)	6.81^c	2.48^g	2.26^b	168^c	570^f	359.5^f
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	6.85^b	2.86^b	1.72^e	139.95^d	586^e	434.60^b
T_4 : *Bacillus* sp. (KAU ref.culture)	6.85^b	3.30^a	1.68^e	139.95^d	530^h	388.5^d
T_5 : *Trichoderma* sp. (KAU ref.culture)	6.67^f	2.80^c	1.97^c	195.95^b	535^g	438^b
T_6 : *Bacillus* sp. + *Trichoderma* sp.	6.77^d	2.69^{de}	1.83^d	224.10^a	635^c	379^e
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	6.94^a	2.58^f	1.56^f	195.98^b	499^i	391.16^d
T_8 : Package of practices (KAU, 2011)	6.69^e	2.87^b	1.9^d	168^c	640^c	387.5^d
T_9 : PGPR Mix – II @ 2.5 kg / ha	6.87^b	2.71^d	2.47^a	195.95^b	660.5^b	358^f
T_{10} : Absolute control	6.88^c	2.76^d	1.97c	139.95^c	680^a	634.5^a
CD (5 %)	0.036	0.035	0.076	7.0	7.48	5.99

Each value represents mean of three replications Initial pH =7.09 Initial organic carbon =3.05 %

Electrical conductivity = 0.47 dSm^{-1} Available nitrogen = 373.74 $mg\ kg^{-1}$

Available phosphorus = 72.70 $mg\ kg^{-1}$ Available potassium = 917.09 $mg\ kg^{1}$

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR Mix – II @ 2.5 kg / ha

harvest, the highest available P was recorded in T_{10} (Control) with 680 mg kg^{-1} and the lowest available P content was in T_7 (Carbendazim + Mancozeb) with 499 mg kg^{-1}.

4.12.1.8.6. Available potassium

Analysis of data revealed significant differences among the treatments (Table 30). The initial available potassium content of the potting mixture was 917.09 mg kg^{-1}. At, the highest available K content of the potting mixture was recorded in T_{10} (Control) with 634.5 mg kg^{-1} and the lowest available K content was recorded in T_9 (PGPR Mix- II) with 358 mg kg^{-1}, which was on par with T_2 (TCH+BCH) with 359.5 mg kg^{-1}.

4.13. Population of *Trichoderma, Bacillus* and *Rhizobium* at the time of flowering and final harvest

4.13.1. Population of *Trichoderma* sp. in the potting mixture

The maximum population of *Trichoderma* sp. at the time of flowering was recorded in T_1 (TPZ+BPN) (46.2×10^4 cfu g^{-1}) followed by T_2 (TCH+BCH) (39.5×10^4 cfu g^{-1}). The lowest *Trichoderma* sp. population was recorded in T_6 (*Bacillus* sp.+*Trichoderma* sp.) (25.2×10^4 cfu g^{-1}) (Table 31).

At harvest, *Trichoderma* sp. population was maximum in T_1 (TPZ+BPN) (3.6×10^2 cfu g^{-1}) followed by T_6 (*Bacillus* sp. + *Trichoderma* sp.) (3.1×10^2 cfu g^{-1}). The lowest *Trichoderma* sp. population was recorded in T_3 (TMT+BML) (2.1×10^2 cfu g^{-1}).

4.13.2. Population of *Bacillus* sp. in the potting mixture

The maximum population of *Bacillus* sp. at the time of flowering was recorded in T_3 (TMT+BML) (38.1×10^4 cfu g^{-1}) followed by T_1 (TPZ+BPN) (35.2×10^4 cfu g^{-1}). The lowest *Trichoderma* sp. population was recorded in T_6 (*Bacillus* sp. + *Trichoderma* sp.) (22.7×10^4 cfu g^1) (Table 32).

Table 31. Population of *Trichoderma* sp. in the potting mixture at the time of flowering and final harvest

Treatments	Population		
	Initial population in the inoculum (x 10^7 cfu g^{-1})	At flowering stage (50 DAS) (x 10^4 cfu g^{-1})	At harvest (100 DAS) (x 10^2 cfu g^{-1})
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	1.9 (3.27)	46.2^{a} (5.66)	3.6^{a} (4.55)
T_2 : Biofilm based *Trichoderma* sp.(TCH) + *Bacillus* sp. (BCH)	2.6 (3.41)	39. 5^{b} (5.59)	2.6^{bc} (4.41)
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	2.4 (3.38)	41.3^{b} (5.61)	2.1^{c} (4.32)
T_4 : *Bacillus* sp. (KAU ref.culture)	0.0 (0.71)	0.0 (0.71)	0.0 (0.71)
T_5 : *Trichoderma* sp. (KAU ref.culture)	5 (3.69)	31.7^{c} (5.50)	3.1^{ab} (4.49)
T_6 : *Bacillus* sp. + *Trichoderma* sp.	5 (3.69)	25.2^{d} (5.40)	2.8^{abc} (4.44)
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	0.0 (0.71)	0.0 (0.71)	0.0 (0.71)
T_8 : Package of practices (KAU, 2011)	0.0 (0.71)	0.0 (0.71)	0.0 (0.71)
T_9 : PGPR mix – II @ 2.5 kg / ha	0.0 (0.71)	0.0 (0.71)	0.0 (0.71)
T_{10} : Absolute control	0.0 (0.71)	0.0 (0.71)	0.0 (0.71)

Each value represents mean of seven replications

Figures in the parenthesis indicate log transformed values   DAS- Days after sowing

T_1 –T_7: Organic Package (KAU, 2009) T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR mix – II @ 2.5 kg / ha

Table 32. Population of *Bacillus* sp. in the potting mixture at the time of flowering and final harvest

Treatments	Population		
	Initial population in the inoculum (x 10^8 cfu g^{-1})	At flowering stage (50 DAS) (x 10^4 cfu g^{-1})	At harvest (100 DAS) (x 10^2 cfu g^{-1})
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	2.1 (3.32)	35.2^{b} (5.46)	3.1^{ab} (4.49)
T_2 : Biofilm based *Trichoderma* sp.(TCH) + *Bacillus* sp. (BCH)	2.6 (3.41)	25.3^{d} (5.40)	4.1^{a} (4.61)
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	1.9 (3.27)	$38. 1^{a}$ (5.58)	4.9^{a} (4.69)
T_4 : *Bacillus* sp. (KAU ref.culture)	2 (3.3)	27.3^{c} (5.43)	3.6^{ab} (4.55)
T_5 : *Trichoderma* sp. (KAU ref.culture)	0.0 (0.71)	0.0 (0.71)	0.0 (0.71)
T_6 : *Bacillus* sp. + *Trichoderma* sp.	2 (3.3)	22.7^{d} (5.35)	2^{b} (3.30)
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	0.0 (0.71)	0.0 (0.71)	0.0 (0.71)
T_8 : Package of practices (KAU, 2011)	0.0 (0.71)	0.0 (0.71)	0.0 (0.71)
T_9 : PGPR mix – II @ 2.5 kg / ha	0.0 (0.71)	0.0 (0.71)	0.0 (0.71)
T_{10} : Absolute control	0.0 (0.71)	0.0 (0.71)	0.0 (0.71)

Each value represents mean of seven replications

Figures in the parenthesis indicate log transformed values

DAS- Days after sowing

T_1 –T_7: Organic Package (KAU, 2009)

T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR mix – II @ 2.5 kg / ha

At harvest, *Bacillus* sp. population was maximum in T_3 (TMT+BML) (4.9×10^2 cfu g^{-1}) followed by T_1 (TPZ+BPN) (4.1×10^2 cfu g^{-1}). The lowest *Bacillus* sp. population was recorded in T_6 (*Bacillus* sp. + *Trichoderma* sp.) (2×10^2 cfu g^{-1}).

4.13.3. Population of *Rhizobium* sp. in the potting mixture

The maximum population of *Rhizobium* sp. at the time of flowering was recorded in T_2 (TCH+BCH) (3.7×10^4 cfu g^{-1}) followed by T_8 (POP KAU, 2011). The lowest *Rhizobium* sp. population was recorded in T_7 (carbendazim + mancozeb) (1.6×10^4 cfu g^{-1}) (Table 33).

At harvest, the maximum population of *Rhizobium* sp. population was recorded in T_9 (PGPR Mix- II) (1.6×10^2 cfu g^{-1}) followed by T_5 (*Trichoderma* sp.) (1.4×10^2 cfu g^{-1}). The lowest *Rhizobium* sp. population was recorded in T_2 (TCH+BCH) and T_3 (TMT+BML) with 1×10^2 cfu g^{-1}.

4.14. Identification of three most promising *Bacillus* sp.

4.14.1. 16S rRNA sequence analysis

Isolate BCH showed maximum homology (92 %) with *Bacillus subtilis*, BML (91 %) with *Bacillus megaterium* and BPN (91 %) with *Bacillus velezensis*. The three isolates were identified as the culturable accession sharing maximum homology with query sequence (Plates 21-23).

The most promising isolates based on growth promotion, antagonistic activity and biofilm production, the *Bacillus* sp. were identified by 16S rRNA sequence analysis.

Table 33. Population of *Rhizobium* sp. in the potting mixture at the time of flowering and final harvest

	Population	
	At flowering stage (50 DAS) (x 10^4 cfu g^{-1})	At harvest (100 DAS) (x 10^2 cfu g^{-1})
T_1 : Biofilm based *Trichoderma* sp. (TPZ) + *Bacillus* sp. (BPN)	2.6^e (4.41)	ND
T_2 : Biofilm based *Trichoderma* sp.(TCH) + *Bacillus* sp. (BCH)	3.7^a (4.56)	1 (2)
T_3 : Biofilm based *Trichoderma* sp. (TMT) + *Bacillus* sp. (BML)	2.6^{de} (4.41)	0.0 (0.71)
T_4 : *Bacillus* sp. (KAU ref.culture)	3.1^c (4.49)	1 (2)
T_5 : *Trichoderma* sp. (KAU ref.culture)	2.7^d (4.43)	1.4 (2.14)
T_6 : *Bacillus* sp. + *Trichoderma* sp.	3.1^c (4.49)	1.2 (2.09)
T_7 : Carbendazim + Mancozeb (2 g / l as soil drenching)	1.6^f (4.20)	0.0 (0.71)
T_8 : Package of practices (KAU, 2011)	3.3^b (4.51)	0.0 (0.71)
T_9 : PGPR mix – II @ 2.5 kg / ha	2.9^f (4.46)	1.6 (2.20)
T_{10} : Absolute control	0.0 (0.71)	0.0 (0.71)

Each value represents mean of seven replications

Initial population in the inoculum – 4.4 x 10^7 cfu g^{-1}

Figures in the parenthesis indicate log transformed values

DAS- Days after sowing

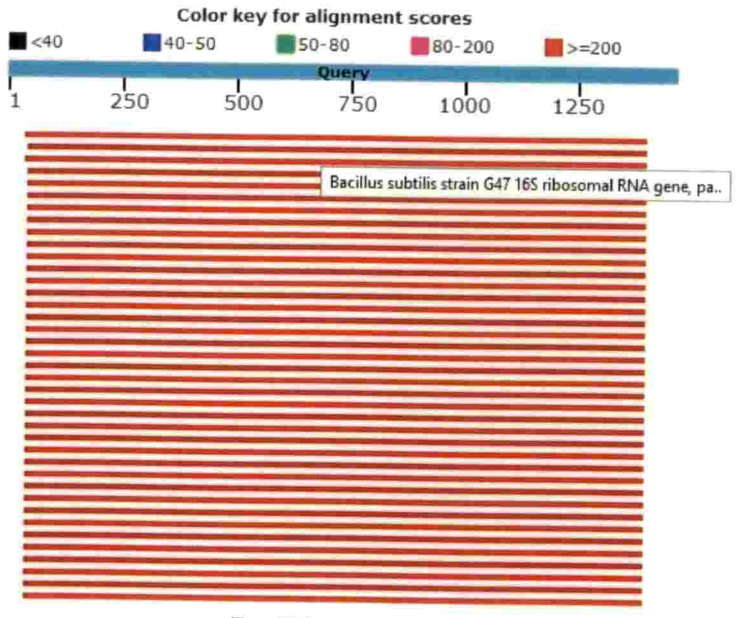
T_1 –T_7: Organic Package (KAU, 2009)

T_8 : Package of practices (KAU, 2011)

T_9 : Organic Package (KAU, 2009) + PGPR mix – II @ 2.5 kg / ha

AGCCGCCGGTCCCCCCTGGGTATTCATGATTGCCCGGGACGGAGGATGGGGGCTTGCTCTTGATGTTAGCGGCGGACGGGTGAGT
AACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGAAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTC
AAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCA
ACGATGCGTACCCGACCTGAGAGGTGATCGCCACACTGATGGACGGCAATCTACGGAGCAGCGTAGGGATCTCCGCAATGGACGA
AGTCTGACGAGCAACGACGCGTGAGTGATGAAGTTTTCGATCGTAAAACTCTGTGTAGGAAGAACAAGTACCCGTCGAATAGGGC
GGTACCTGGACGGTACCTAAACAGAAAGCCACGGCTAATACGTGCCAGCAGCGCGGTATACGTATGGGGCAAGCGTGTCCGGAAT
TATTAGGCGTAAAGAGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAACCCCCGCTCAACCGGGGAGGGTCATTGGAAACTGGG
GAATTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCACTGGCGAAGGC
GACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTACTCCACGCCGTAAAC
GATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAACTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAG
ACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCACGCGAAAAACCTTACCAGG
TCTTGACATCCTCTGAAAATCCTAAAGATAGACGTCCCCTTCGGGGGCAGAGGGACAGGTGGTGCATGATTGCCACCAGCTCGTGT
CGAGAGATGTAGGGATAAATCCCACAACGAGCACAACCCTTGTCTTAATTGCCAACATTCAGTTGGGCACTCTAAGGTGACTGCCG
GTGACAAACCGGAGGAAGGTGGGGAGGACGTCAAATCATCATGCCCCTTATGACCTGGGGTACACACGTGCTACAATGGACAGAA
CAAAGGGTAGAGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTTTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAG
CTGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTT
TGTAACACCCGAAGTCGGTGAGGTAACCTTTAGGACCTTTTACTTTGAACCCGGGACAGGTCCGGGGGGACTTAACAGGTACAGA+
AAGTTACCGCCACCGG

A. Partial sequence of 16S rRNA amplicon



B. Blastn output

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus subtilis strain G47 16S ribosomal RNA gene, partial sequence	2124	2124	92%	0.0	95%	JX293305.1
Bacillus subtilis strain G19 16S ribosomal RNA gene, partia	2122	2122	92%	0.0	95%	KX343978.1
Bacillus subtilis strain XJ-4J 16S ribosomal RNA gene, parti	2122	2122	92%	0.0	94%	HM631838.1
Bacillus subtilis strain p1 16S ribosomal RNA gene, partial sequence	2121	2121	92%	0.0	95%	MF073326.1
Bacillus sp. strain WC5 16S ribosomal RNA gene, partial sequence	2121	2121	92%	0.0	95%	JN975953.1
Bacillus subtilis strain X502 16S ribosomal RNA gene, partial sequence	2121	2121	92%	0.0	95%	KU240496.1
Bacillus subtilis strain GQJK6 16S ribosomal RNA gene, partial sequence	2121	2121	92%	0.0	95%	KY952699.1
Bacillus subtilis strain GQJK4 16S ribosomal RNA gene, partial sequence	2121	2121	92%	0.0	95%	KY952698.1
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

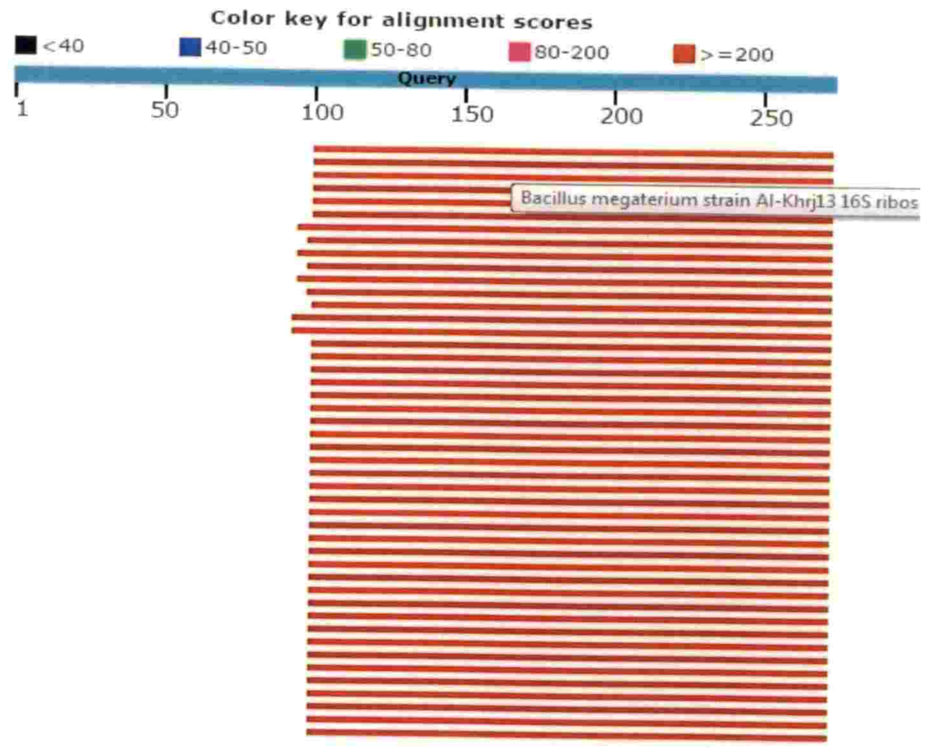
Go to alignment for Bacillus subtilis strain G47 16S ribosomal RNA gene, partial sequence > gb|JX849066.1| Bacillus sp. M47 16S ribosomal RNA gene, partial sequence

C. Sequence showing homology

Plate 21. Partial sequence analysis of isolate BCH

AAACGTGGAACTCTTAGGAATGTCCCGCGGCACCTGCCAGTTAAAATTAA
AAGGGGGTACCCCCCCCCCCTGACTTCGGTAAATATAATAGGGGGGGCG
CGACGGGCGGTGTGTAAAGGCCCGATAACGTATTCGCCGCGGCATGCTG
ATCCGCGATTACTAGCGATTCCAGCTTCTGTAGGCGTGTTGCAGCCTAAA
TCCGAACTGAGAATTTTTTTATGGGATTGGCTTGACCTCGCGGGCTTGCA
GTACTTTGTTTATCCATTTGAGCACT

A. Partial sequence of 16S rRNA amplicon



B. Blastn output

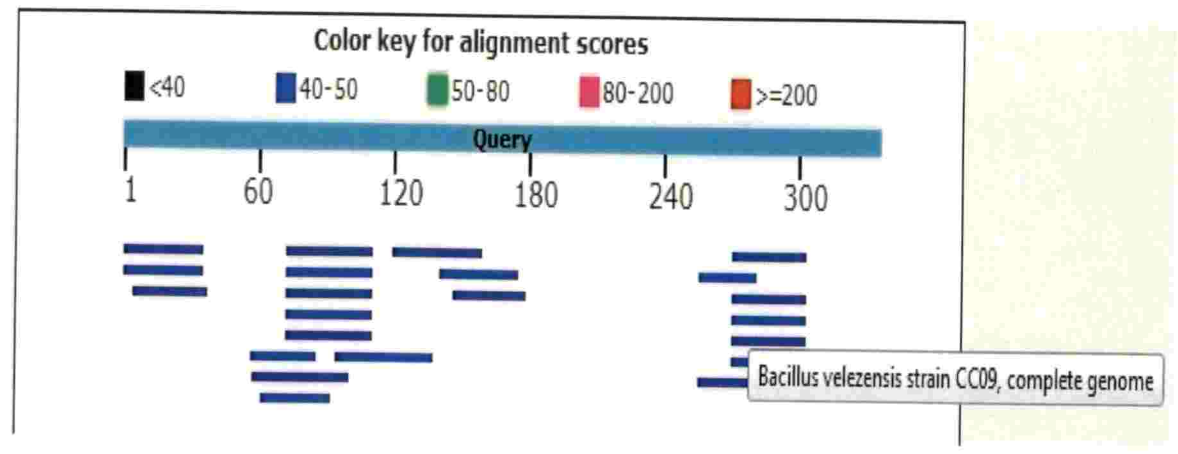
Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus megaterium strain Al-Khrj13 16S ribosomal RNA gene, partial sequence	235	235	63%	4e-58	91%	KY123866.1
Bacillus sp. IARI-SL-28 16S ribosomal RNA gene, partial sequence	235	235	63%	4e-58	91%	JX645213.1
Bacillus megaterium strain BAB256 16S ribosomal RNA gene, partial sequence	235	235	63%	4e-58	91%	JF792053.1
Bacillus aryabhattai strain OFB-3 16S ribosomal RNA gene, partial sequence	235	235	63%	4e-58	91%	HM439455.1
Uncultured bacterium clone SR36 16S ribosomal RNA gene, partial sequence	235	235	63%	4e-58	91%	DQ298290.1
Uncultured bacterium clone 4 sp AR 16S ribosomal RNA gene, partial sequence	233	233	63%	1e-57	90%	KX344502.1
Bacillus megaterium strain 22-A1 16S ribosomal RNA gene, partial sequence	233	233	64%	1e-57	90%	KU647215.1

C. Sequence showing homology

Plate 22. Partial sequence analysis of isolate BML

GACAAAAAAAGTTCCGACTAATATAACAAAAAGAAATTCGTCTAAGCGGTGAGAGACTTT
TTATCCAGCAGTCAGATTTTTTTTATGAGTTTCGTATAAAAGCGAGCGGTTAAAGGTATC
AATAAGTAAATTACTCCTTGAACGCAGTTTTTGATAAAAGATTGTACCCAAACATGACCG
TCCTCCGACGCTACTTAACCCCGGGACCCATAAAGTATCGCCACACATAGATGAGGCCCA
GAGTGGATTATCCATTATAGTCTTCATGCCCCAATTTTTGTTATTGCCCTGGCAGTTTAT
CTTAGAAGTTAGTGACGAGAGGTCTCGCACCGGAGCT

A. Partial sequence of 16S rRNA amplicon



B. Blastn output

Bacillus velezensis strain CC09, complete genome	42.8	42.8	9%	6.2	91%	CP015443.1
Bacillus amyloliquefaciens strain B15, complete genome	42.8	42.8	9%	6.2	91%	CP014783.1
Bacillus amyloliquefaciens UMAF6639, complete genome	42.8	42.8	9%	6.2	91%	CP006058.1
Bacillus sp. Pc3, complete genome	42.8	42.8	9%	6.2	91%	CP010406.1
Brugia pahangi genome assembly B_pahangi_Glasgow, scaffold BPAG_scaffold0000643	42.8	42.8	9%	6.2	88%	LK965770.1
Angiostrongylus cantonensis genome assembly A_cantonensis_China, scaffold ACAC_scaffold0000281	42.8	42.8	10%	6.2	89%	LK945876.1

C. Sequence showing homology

Plate 23. Partial sequence analysis of isolate BPN

5. DISCUSSION

Microbial inoculants are the important components of both integrated disease and integrated nutrient management systems. They are known to help in the management of plant diseases and improve the plant growth by mineral uptake, disease suppression, nitrogen fixation, or phytohormone production (He *et al*., 2007). Among the microbial antagonists, *Trichoderma* sp. and *Bacillus* sp. are the most successful biocontrol agents which have been commercially exploited for the management of plant diseases. *Trichoderma* sp. is widely used for the management of fungal diseases and is a potential plant growth promoter (Harman *et al.,* 2004). Similarly, *Bacillus* sp. is considered to be an important biocontrol agent as well as PGPR (Bais *et al*., 2004). Several studies have indicated that microbial consortia are effective in enhancing the efficiency of antagonists and growth promotion due to multiple action (Srinivasan *et al*., 1996). However, one of the major constraints in the use of conventional bioinoculants is poor survivability of the introduced microorganisms in the soil due to various environmental stress factors (Bashan and Levanony, 1998). The ability of the introduced strains to colonize roots and survive in soil are either limited/restricted which reduces the efficiency of the antagonists (Bolwerk *et al.,* 2003). Hence, there is a need to enhance the survivability of the introduced bioagents to get the desired benefit.

Cowpea is an important legume crop and inexpensive source of vegetable protein widely cultivated in Kerala and the climatic conditions are highly favorable for the cowpea cultivation in Kerala. However, one of the major constraints is the occurrence of fungal diseases, particularly collar rot and root rot caused by *Rhizoctonia solani* and *Pythium aphanidermatum* respectively. Eventhough, the pathogen can be controlled by microbial antagonists, the survivability of the inoculated biocontrol agents in the soil are very poor.

Biofilm is an aggregation of microorganisms that forms a protective adhesive matrix which protects the microorganisms from the external factors like high temperature, antibiotics, salinity *etc*. (Costerton *et al*., 1995). Biofilms established on plant roots acts as a sink for the nutrients which reduces the root exudates for the

pathogen colonization and subsequent stimulation (Weller and Thomashow, 1994). It has already been reported that the synergistic interaction between two bioagents enhances biofilm formation and increases resistance to antimicrobial agents. Hence, a study was undertaken to evaluate biofilm based *Trichoderma* sp. and *Bacillus* sp. for the management of rot diseases and growth promotion in cowpea.

5.1. Enumeration of *Trichoderma* sp. and *Bacillus* sp.

In the present studies, native isolates of *Trichoderma* sp. and *Bacillus* sp. were isolated from major cowpea growing areas of Thrissur district. The population of *Trichoderma* sp. varied among the locations (Table 2). The highest population of *Trichoderma* sp. (4.8×10^3 cfu g^{-1}) (TCH) was recorded in healthy cowpea of Chellakkara location whereas, the *Bacillus* sp. population was highest in healthy cowpea of Pananchery location (4.48×10^5 cfu g^{-1}) (BPN). The results indicated that the healthy plants of cowpea harbored more population of antagonists compared to the rhizosphere of infected plants. Da Mota *et al.,* (2008) reported that soil microorganisms vary in their population and are influenced by high temperature, dryness/heavy rainfall in tropical countries. Out of ten locations, nine *Trichoderma* sp. and five *Bacillus* sp. were obtained in the present studies. The isolates obtained from different locations were characterized and identified at genus level. Characterization revealed *Trichoderma* sp. colonies as whitish green to dark green and the hyphae were septate and hyaline under microphone (Table 4). It was also found that conidiophores were hyaline and conidia were green in colour. These characters were compared with the standard keys (Shah *et al*., 2012) and found to be similar to the *Trichoderma* sp. The results are in agreement with the previous literature (Shah *et al*., 2012) and hence, the isolates were tentatively assigned to the genus *Trichoderma.* Similarly, the *Bacillus* sp. characters of Gram's reaction, endospore staining, shape and size were compared with the standard keys mentioned in Bergey's Manual of Determinative Bacteriology (Holt, 2004). Based on the standard keys, the isolates were tentatively assigned to the genus *Bacillus*. It is well known that the beneficial microorganisms improve the plant growth and control diseases (Peoples *et al*., 1995). Therefore, nine *Trichoderma* sp. and five *Bacillus* sp.

obtained in the present studies were screened for growth promoting and antagonistic activities.

5.2. *In vitro* screening of *Trichoderma* sp. and *Bacillus* sp. for antagonistic activities and plant growth promoting activities

Out of nine *Trichoderma* isolates, two isolates (TCH-1 and TPZ) were positive for HCN production (Table 9) and five isolates (TCH-1, TMT, TMS, TP and TCK-1) for ammonia production (Table 10). *Trichoderma* sp. is the most commonly used biocontrol agent for the management of several phytopathogenic fungi and plays a multiple role as biopesticide, bioherbicide and plant growth promoter (Sriram *et al.*, 2013). Bharadwaj and Gupta (1987) reported that *in vitro* evaluation using *Trichoderma viridae, Trichoderma harzianum, Trichoderma hamatum* and *Trichoderma harzianum* against *Pythium aphanidermatum* and *Fusarium oxysporum* were inhibitory to pathogens through various mechanisms. These results are in agreement with present study where, the *Trichoderma* sp. exhibited various mechanisms such as HCN production (Plate 7) and ammonia production (Plate 8). Among the *Bacillus* sp. two isolates were found to be positive for HCN production (Table 9), five isolates were positive for ammonia production (Table 10), in the present study. It has already been reported by several authors that the antagonists are able to control the pathogen through, HCN production (Reetha *et al.*, 2014), mycoparasitism (Verma *et al.*, 2007), antibiosis (Pan *et al.*, 2013) and competition (Elad, 2000) by both *Trichoderma* sp. and *Bacillus* sp. which is in agreement with present studies.

Indole acetic acid is an important hormone, essential for growth and development of the plants. Therefore, the isolates of *Trichoderma* sp. and *Bacillus* sp. obtained were screened for IAA production. The studies revealed that four isolates of *Trichoderma* sp. were positive for IAA production (Table 6). In a similar study, Resende, *et al.* (2014) reported that *Trichoderma asperellum* not only solubilized iron phosphate and ammonium phosphate but also synthesized indole acetic acid indicating that *Trichoderma* sp. could be a potential plant growth promoter. Ranasingh *et al.* (2006) reported that *Trichoderma* strains are an effective

biocontrol agent for the management of plant diseases and they have the ability to increase the root growth and development, crop productivity, resistant to abiotic stresses and uptake and use of nutrients. It was also reported that production of metabolites from different *Trichoderma* strains depends on ecological factors which might be the reason for the strains to show varying effects on the pathogens (Henis, 1984; Papavizas, 1985) and these results are in agreement with the present studies. Among *Bacillus* sp., two isolates (BCH and BMS) were positive for IAA production. In the present studies, *Bacillus* sp. and *Trichoderma* sp. varied in their mechanisms under *in vitro*. This indicated that both *Bacillus* sp. and *Trichoderma* sp. produced metabolites differently under different conditions of soil. These results are in agreement with Papavizas, (1985) who reported that the production of metabolites from different *Trichoderma* strains were influenced by the ecological factors.

Phosphorus is a major growth limiting nutrient and there are no large scale resources in the atmosphere which can be made biologically available. Strains of *Pseudomonas*, *Bacillus*, *Rhizobium*, *Enterobacter* and *Aspergillus* have been reported to be the most efficient P solubilizers. Similarly, the *Trichoderma* is also known to be a P solubilizing fungus. Hence, *Trichoderma* sp. and *Bacillus* sp. obtained in the present studies were screened for P solubilisation. Among the *Trichoderma* sp., four isolates (TPZ, TCH-1, TMS and TMT) recorded P solubilisation out of nine different *Trichoderma* isolates (Table 8). Most of the P in soil is present in the form of insoluble phosphates and cannot be utilized by plants (Pradhan and Sukla, 2005). The use of P solubilizing microorganisms as inoculants increases the P uptake by the plants. Among the *Bacillus* sp., only two isolates (BML and BPN) were positive for P solubilisation (Table 7) and the highest P solubilisation index was recorded by BPN isolate (29.45 %) (Plate 6). In the present study, most of the *Bacillus* sp. and *Trichoderma* sp. isolates solubilized phosphorus under *in vitro* conditions. In a similar study, Rodriguez and Fraga (1999) reported P solubilisation in *Pseudomonas striata* and *Bacillus polymyxa* with 156 and 116 mg L^{-1} respectively which is in agreement with the present study.

In order to develop an efficient microbial antagonist, it is important to screen the antagonistic efficiency of isolates against pathogens under *in vitro* conditions. In the present study, *Trichoderma* isolates showed antagonistic activity against both *Pythium aphanidermatum* and *Rhizoctonia solani* (Table 12). The isolate of *Trichoderma* sp. (TCH-1) recorded highest inhibition (57.7 %) against *Pythium aphanidermatum* under *in vitro* and the same isolate also recorded highest per cent inhibition (51.1 %) against *Rhizoctonia solani.* The antagonistic activity of *Trichoderma* sp. is due to the production of various secondary metabolites which act as inhibitors to various plant pathogens. In the present studies, *Trichoderma* sp. were found to be effective against *Rhizoctonia solani* (Plate 11) and *Pythium aphanidermatum* (Plate 12). These results are in agreement with Khare *et al.* (2010) who reported that the mutant strain of *Trichoderma viridae* (1433) showed significant antagonistic activity against *Pythium aphanidermatum* by the production of volatile and non-volatile metabolites. Similarly, Pan *et al.* (2013) also reported that different isolates of *Trichoderma* sp. were effective against *Rhizoctonia solani, Sclerotium rolfsii, Fusarium ciceri* and *Machrophomina phaseolina* under *in vitro* conditions which is due to the production of both volatile and non-volatile inhibitors of *Trichoderma* sp.

Among the *Bacillus* sp., none of the isolates showed antagonistic activity against *Rhizoctonia solani* (Plate 10) and *Pythium aphanidermatum* (Plate 12). *Bacillus* sp. is most abundant in rhizosphere and the PGPR activity of some strains have been known for many years (Gutierrez *et al.*, 2003). Different *Bacillus* sp. were reported to be effective biocontrol agents in green house or field trials. However, no correlation has been found between the *Bacillus* isolates in the form of metabolites that are effective against *Fusarium oxysporum* on diggerent media *in vitro* (Grosch *et al.*, 1998). In another study, it has been reported that some of the *Bacillus* sp. failed to show antagonistic activity against *Pythium aphanidermatum* which might be either due to lack of ability to produce or insufficient production of antimicrobial compounds which are inhibitory to *Pythium aphanidermatum* (Jayaseelan *et al.*, 2012) which is in agreement with the present study where, *Bacillus* sp. did not show any antagonistic activity against *Pythium aphanidermatum* and *Rhizoctonia solani.*

5.3. Screening of *Bacillus* sp. for biofilm production

The application of microbial antagonists in soil often do not reproduce their beneficial effect consistently because the survival and establishment of these organisms in rhizosphere soil are influenced by various environmental stresses. Therefore, there is a need to think on alternate approach so as to improve the survivability and efficiency of inoculated microbial antagonists. Microbial biofilms are the communities of microorganisms adhering to abiotic and biotic surfaces and they are embedded in an organic matrix of biological origin which provides structure and stability to the community (Webb *et al.*, 2003). Since biofilms comprise layers of microbial cells, they can play a key role in plant microbe interactions. Microbes in biofilm are sessile and encased in extra cellular polysaccharide matrix which provides protection from environmental stress (Flemming, 2001). So, there is a scope for microbial biofilm production to overcome the poor survival of microbial inoculants under harsh environmental conditions. Therefore, in the present studies, *Bacillus* isolates were screened for biofilm production under *in vitro* conditions using three different methods (Plate 14). Based on the TCP, the BCH isolate (0.060) was the most efficient biofilm producer followed by BPN (0.058) isolate and BML (0.056) isolate (Table 13). Warmink *et al.* (2011) reported that novel microhabitants are formed in soil which determines the ecological success of the inoculated bacteria and enhances the movement and survival of bacteria in soil especially as biofilms. In the present studies, the *Bacillus* sp. were found to be the potential biofilm producers. These results are in agreement with the studies conducted by Bais *et al.* (2004) and Cavaglieri *et al.* (2005) who reported that *Bacillus subtilis* protects roots from plant pathogenic bacteria by biofilm formation, antibiotic and surfactin production which possesses antimicrobial activity against pathogens. Similarly, Timmusk *et al.* (2005) also reported that *Paenibacillus polymyxa* provided protection from pathogens, when it formed biofilms by colonizing *Arabidopsis thaliana.*

5.4. Screening of *Trichoderma* sp., *Bacillus* sp. and *Rhizobium* sp. for mutual compatibility under *in vitro*

Based on the plant growth promoting and antagonistic activities, the three most promising isolates of *Trichoderma* sp. (TCH, TPZ and TMT) and *Bacillus* sp. (BCH, BPN and BML) were selected based on the plant growth promoting traits, antagonistic activities and biofilm production. Application of single biocontrol have certain limitations with regard to consistency and efficacy in different environmental conditions (Gumede, 2008). One of the important strategy to overcome these limitations are by combining two or more beneficial microorganisms in single application as they are able to adapt to different environmental conditions which may complement each other (Kildea *et al.*, 2008). The combination of microorganisms also provides the benefit of broad spectrum activity by the combination of various traits. In this regard, mutual compatibility between *Trichoderma* sp. and *Bacillus* sp. were evaluated in order to explore the possibility of using *Trichoderma* sp. and *Bacillus* sp. in biofilm based inoculum. The results indicated that the *Trichoderma* sp. and *Bacillus* sp. were compatible to each other. These results are in agreement with Fuja *et al.* (2016) who reported that *Bacillus* sp. and *Trichoderma* sp. obtained from forest areas of Brazil were mutually compatible with each other.

In the present studies, *Trichoderma* sp. and *Rhizobium* sp. as well as *Bacillus* sp. and *Rhizobium* sp. were also mutually compatible with each other. These results are in agreement with the results of Jayaraj and Ramabadran (1999) who reported compatibility between *Trichoderma* sp. and *Rhizobium* sp. isolates. However, there are also reports that the growth of *Rhizobium* sp. showed slight inhibition effect on some of the strains of *Trichoderma* sp. (Sethi and Subha Rao, 1968) which is contradictory with the present studies. However, in the present study *Trichoderma* sp. and *Rhizobium* sp. were found to be compatible with each other indicating that further studies are needed to confirm this.

5.5. Standardization of carrier material for *Trichoderma* based *Bacillus* sp. biofilm inoculant production

One of the major limitations to the application of microbial antagonists such as *Trichoderma* sp. and *Bacillus* sp., are the use of suitable carrier material to develop a formulated product (Fravel, 2005). Eventhough, a wide range of formulations of the biocontrol agents have been tested (Kolombet *et al.*, 2008), they are not effective in maintaining the viable cells during storage. Hence, standardization of carrier material for the mass multiplication of *Trichoderma* based *Bacillus* sp. biofilm inoculant was undertaken so as to find out the most suitable carrier material. The studies revealed highest population of *Trichoderma* sp. (29 x 10^5 cfu g^{-1}) and *Bacillus* sp. (19 x 10^5 cfu g^{-1}) in the case of talc powder followed by vermiculite at 90 DAI (Table 14). It has been reported that lentil seeds treated with formulations of *Bacillus subtilis* on glucose, talc and peat significantly enhanced its biocontrol activity against *Fusarium* (Hassan and Gowen, 2006). They also reported that the glucose and talc based formulations were more effective than peat. Bheemaraya *et al.* (2011) also reported highest population of *Trichoderma* sp. in the case of vermicompost (85 x 10^6 cfu g^{-1}) and talc (69.56 x 10^6 cfu g^{-1}). These results are in agreement with the present study where talc powder was found to be the most suitable carrier material for *Trichoderma* sp. and *Bacillus* sp. However, further studies are needed to determine carrier material which will enhance the shelf-life of biofilm based inoculum.

5.6. Evaluation of biofilm based antagonists for growth promotion and disease management in cowpea under pot culture experiment

Three most promising *Trichoderma* based *Bacillus* sp. (TCH+BCH, TPZ+BPN and TMT+BML) biofilm inoculants with talc powder as carrier material were evaluated for their efficiency in disease management and growth promotion in cowpea under pot culture. The experiment was conducted as separate studies with challenge inoculation of *Rhizoctonia solani* and *Pythium aphanidermatum.* In general, the biofilm based inoculant comprising of TCH (*Trichoderma* sp.) + BCH (*Bacillus* sp.) (T_2) was the most promising treatment for the management of collar

rot and growth promotion in cowpea (Plate 21). Biofilm based TCH+BCH (T_2) treatment performed better with respect to early germination (Table 16), plant height (Fig 1), number of leaves (Fig 2), minimum days taken for flowering (54.28) (Fig 3), fresh weight (70.37 g plant^{-1}) and dry weight (11.25 g plant^{-1}) of plants (Fig 4). Microorganisms associated with plants generally protects the hosts against the pathogen. There is a need to use the combination of consortia of microorganisms having different functional attributes. In the present studies, biofilm based inoculant of *Trichoderma* sp. (TCH) and *Bacillus* sp. (BCH) were found to be the most promising one. These results are in agreement with Triveni *et al.* (2015) who reported that *Anabaena* based biofilms which exhibited superior plant growth promoting and biocontrol traits in various crops. It has also been reported that combination of strains of bacteria/fungal antagonists are more efficient in biocontrol than monocultures (Nakkeeran *et al.*, 2005) which is in agreement with present study.

Similarly, in the case of root rot management and growth promotion in cowpea, T_2 (TCH (*Trichoderma* sp.+BCH (*Bacillus* sp.)) treatment was found to be the most promising inoculant. Biofilm based TCH (*Trichoderma* sp.) + BCH (*Bacillus* sp.) (T_2) treatment performed better with respect to early germination (Table 24), plant height (Fig 5), number of leaves (Fig 6), minimum days taken for flowering (52.71) (Fig 7), fresh weight (63.71 g plant^{-1}) and dry weight (10.50 g plant^{-1}) of plants (Fig 8). These results are in agreement with Triveni *et al.* (2015) who reported that Anabaena- *Bacillus subtilis* biofilm treatment recorded significantly higher plant and soil nutrient parameters in cotton crop and reported that biofilmed biofertilizer with multiple useful traits could be beneficial for effective nutrient and pest management in cotton crop.

5.7. Effect of different treatments on nutrient status of the potting mixture before and after the experiment

In the present studies, effect of different on the nutrient status of the potting mixture before and after the experiment were recorded in *Rhizoctonia solani* (Table 23) and *Pythium aphanidermatum* treated plants (Table 30). The pH and nutrient status of the potting mixture revealed decrease in pH, electrical conductivity, organic

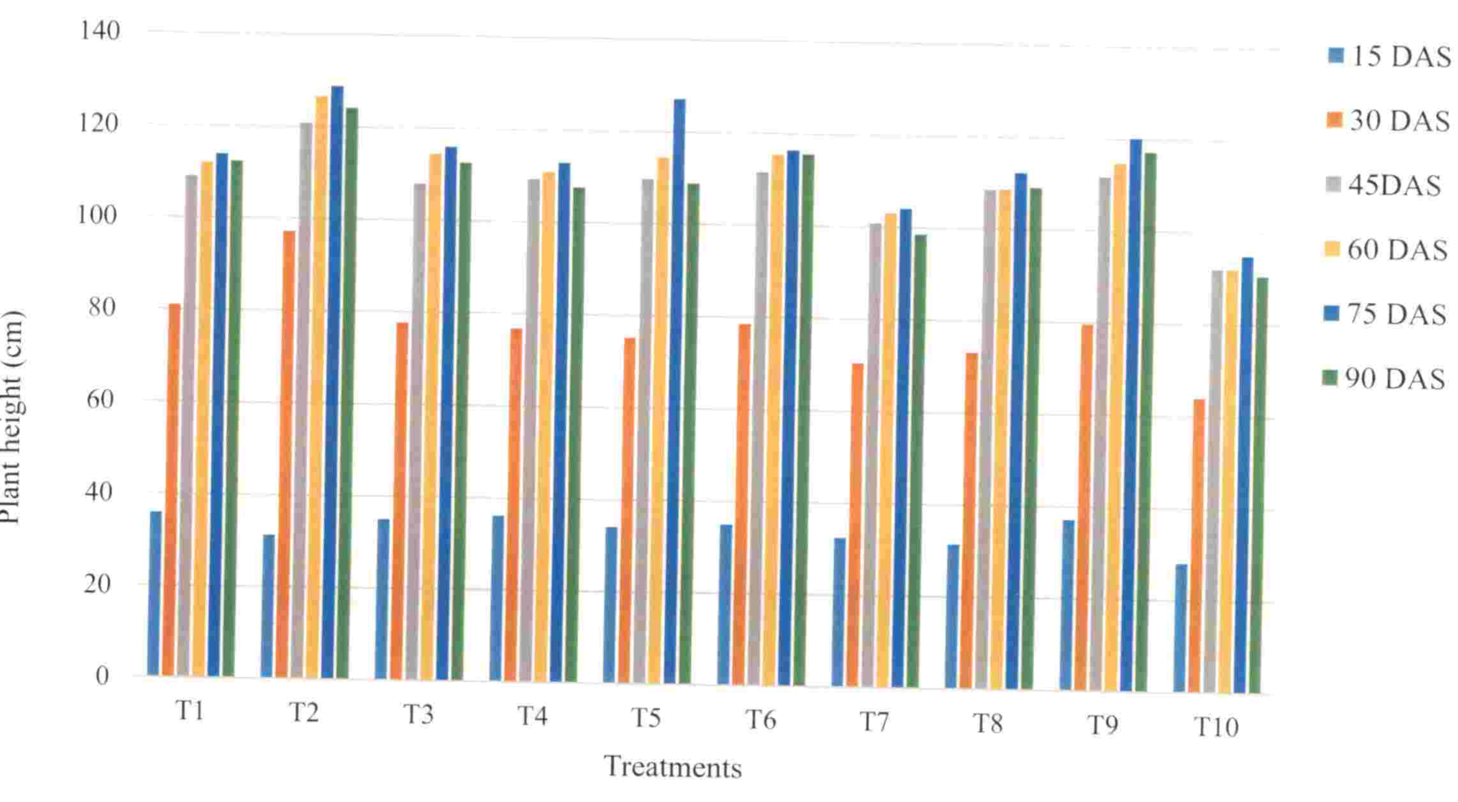


Fig. 1. Effect of different treatments on plant height of cowpea before and after the challenge inoculation with *Rhizoctonia solani* (30 DAS)

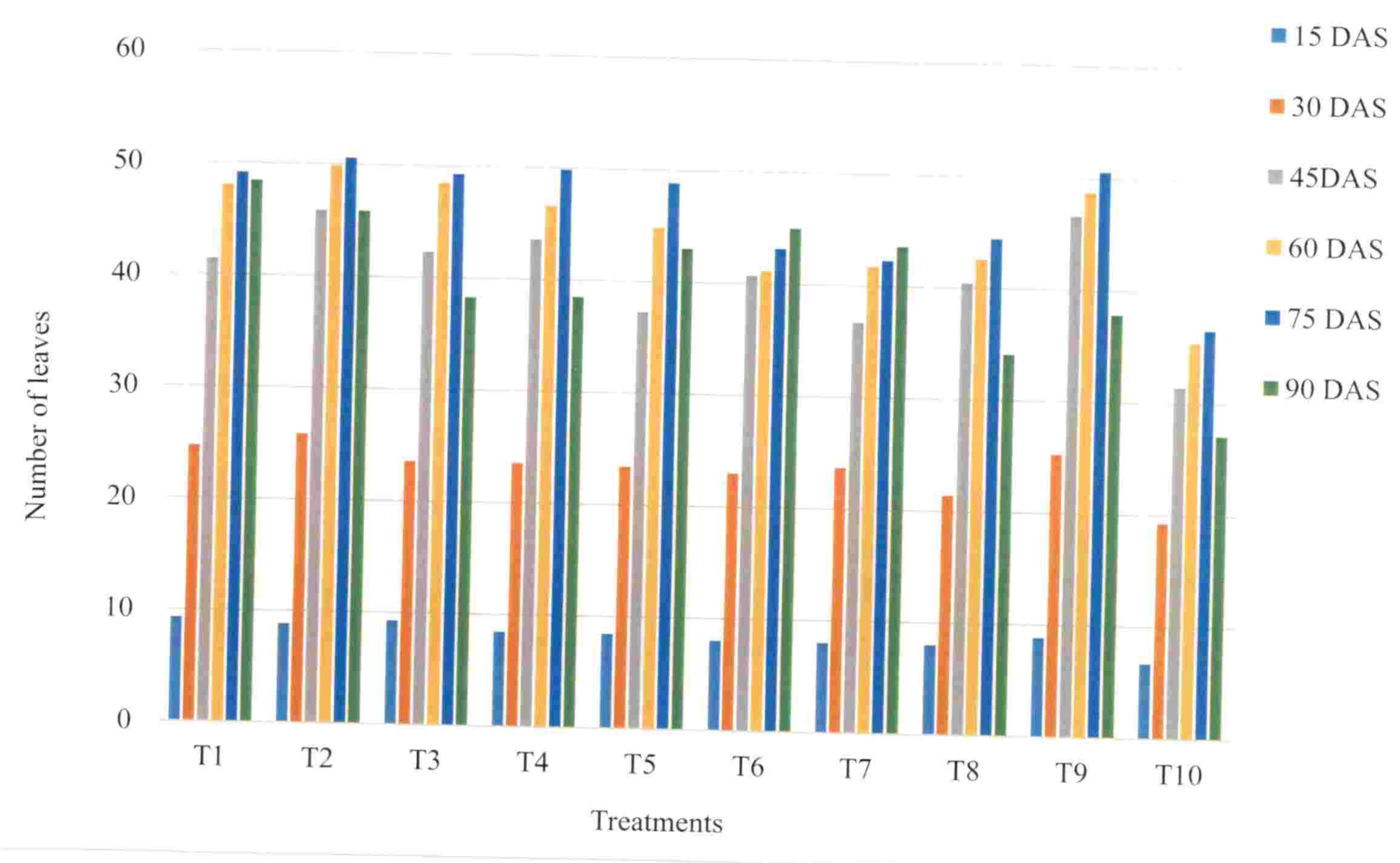


Fig. 2. Effect of different treatments on number of leaves before and after the challenge inoculation with *Rhizoctonia solani* (30 DAS)

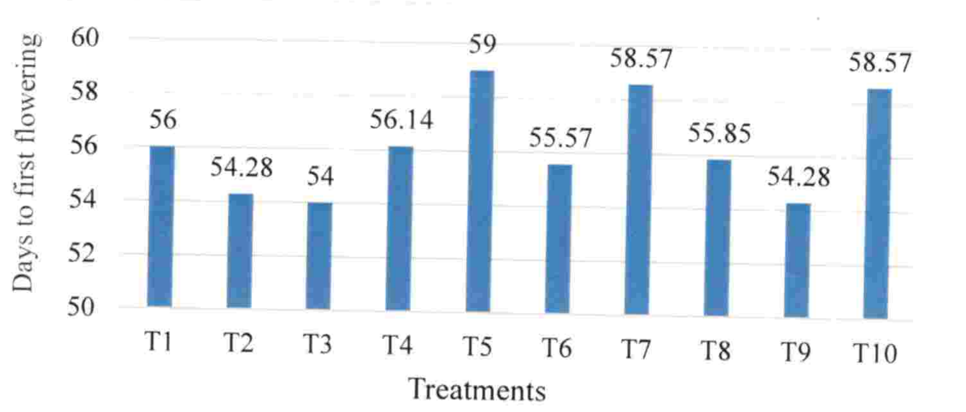


Fig. 3. Effect of different treatments on the number of days taken for first flowering in cowpea after challenge inoculation with *Rhizoctonia solani* (30 DAS)

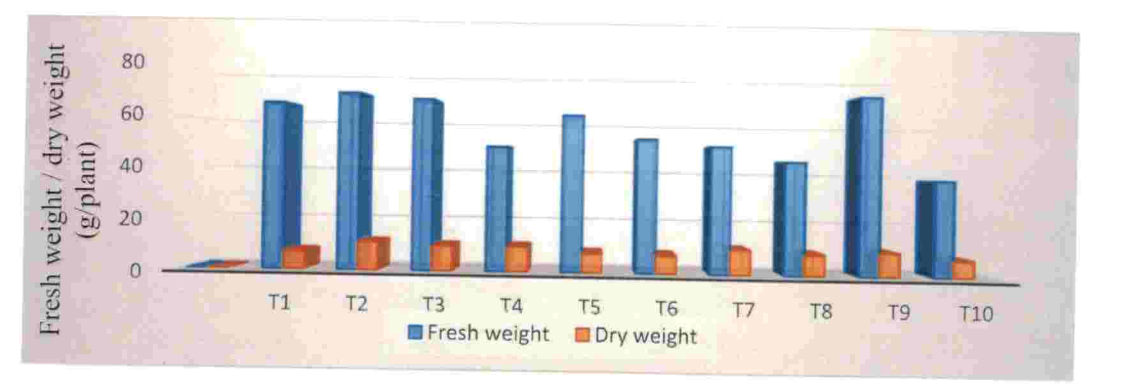


Fig. 4. Effect of different treatments on fresh and dry weight of cowpea after challenge inoculation with *Rhizoctonia solani* (30 DAS)

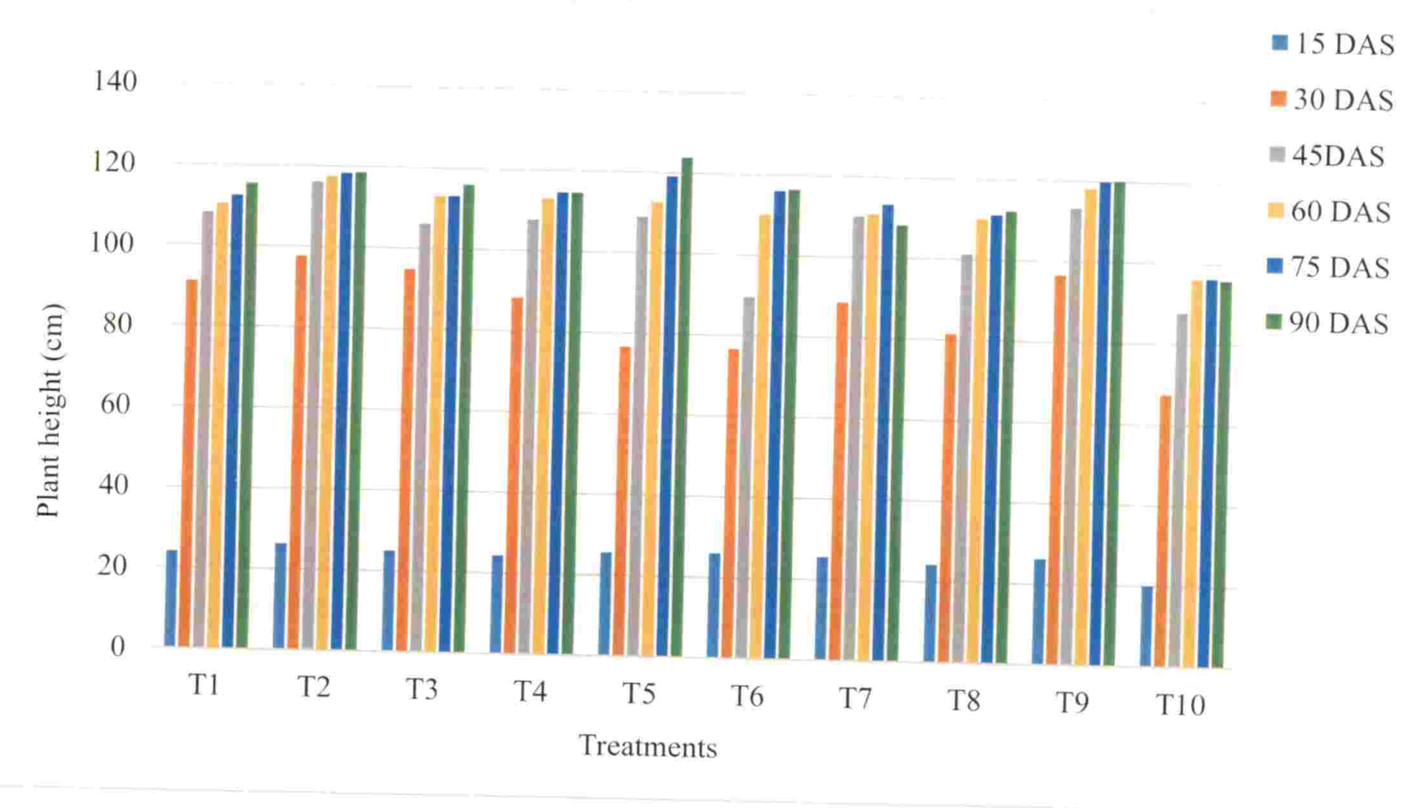


Fig. 5. Effect of different treatments on plant height of cowpea before and after challenge inoculation with *Pythium aphanidermatum* (30 DAS)

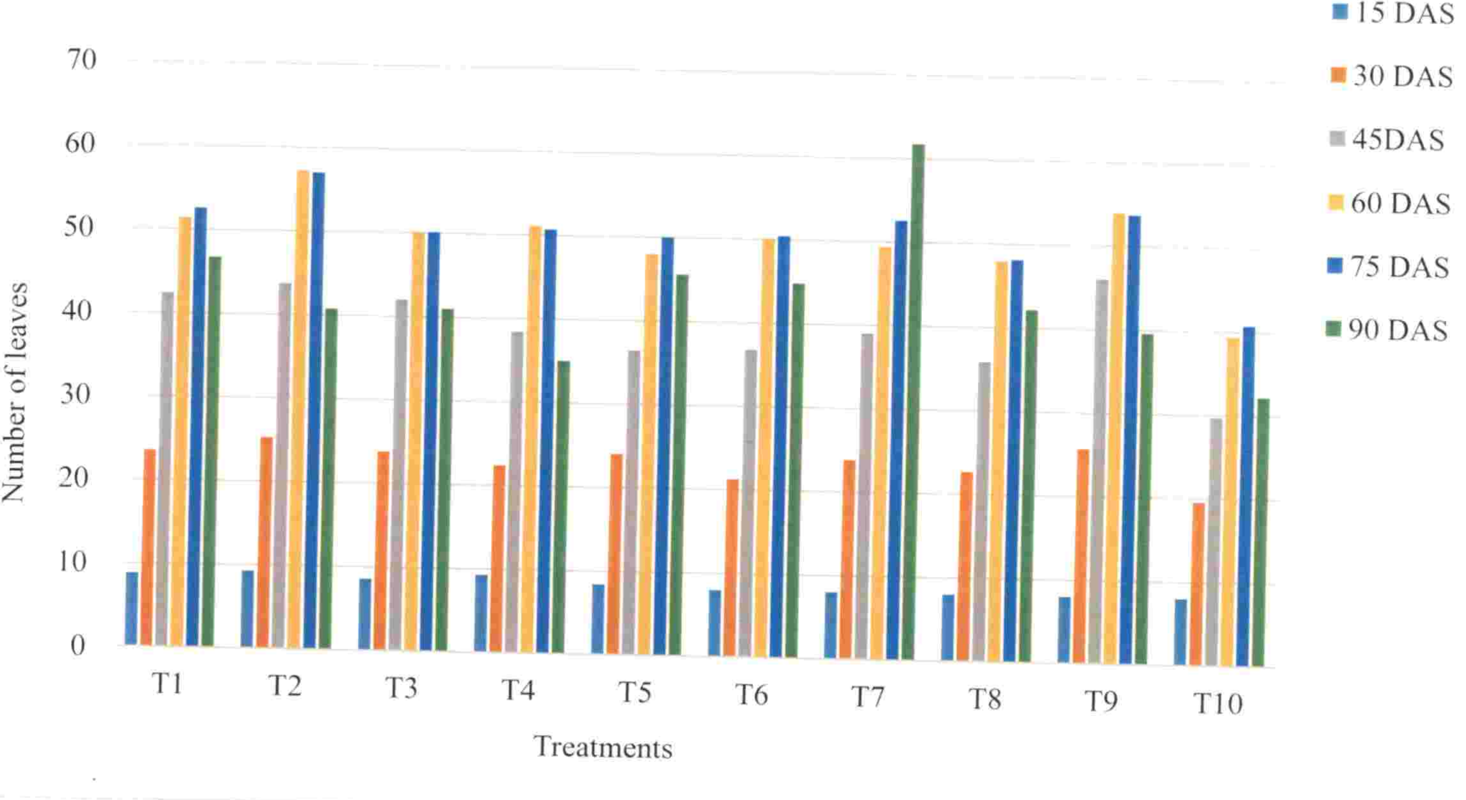


Fig. 6. Effect of different treatments on number of leaves before and after challenge inoculation with *Pythium aphanidermatum* (30 DAS)

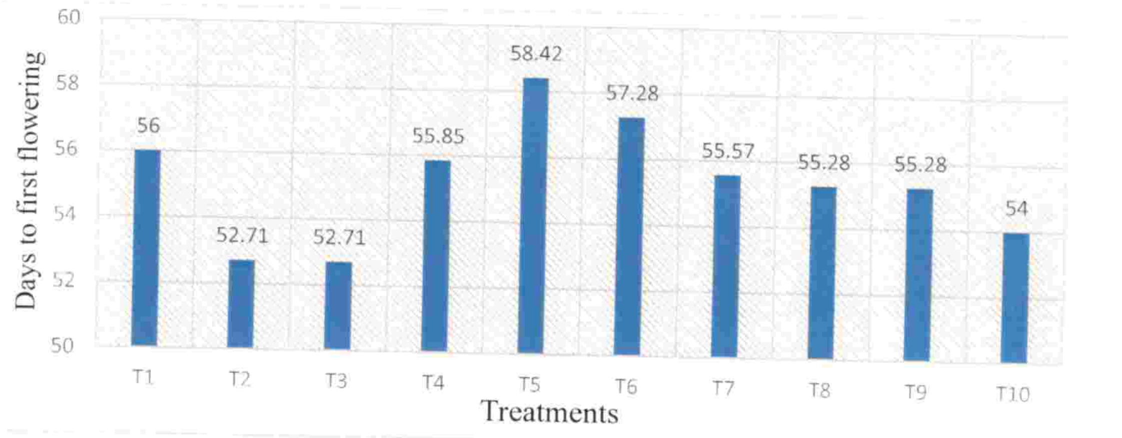


Fig. 7. Effect of different treatments on number of days taken for first flowering in cowpea after challenge inoculation with *Pythium aphanidermatum* (30 DAS)

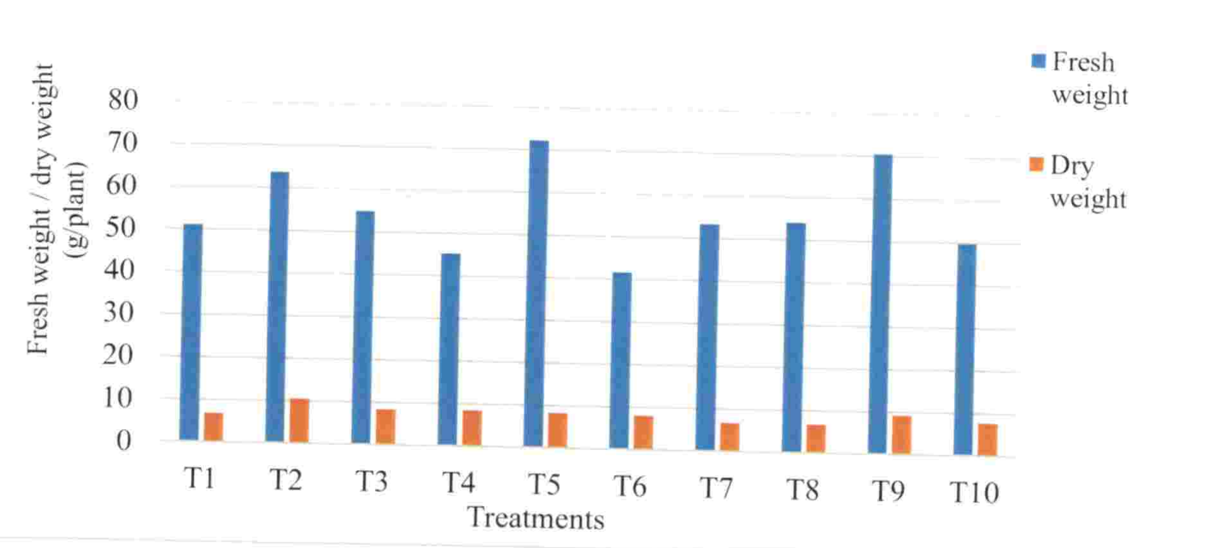


Fig. 8. Effect of different treatments on fresh and dry weight of cowpea after challenge inoculation with *Pythium aphanidermatum* (30 DAS)

carbon and available N, P and K after the experiment. However, the highest pH (7.1) was observed in the case of biofilm based *Trichoderma* sp. (TCH) and *Bacillus* sp. (BCH) while the lowest pH was observed in T_9 (PGPR Mix- II) (Table 23). As the potting mixture consisted of FYM, soil and sand (1:1:1), the marginal decrease in the pH might be due to the organic acid production during the decomposition of organic manure as well as the enzyme and hormonal effect of microbial inoculants (Chen *et al.* 2006).

Organic carbon is an important source of nutrients for the microorganisms. The initial organic carbon content in the potting mixture was 3.05 % but, it decreased at the end of the experiment. The maximum organic carbon was recorded in the case of T_2 (TCH (*Trichoderma* sp.) +BCH (*Bacillus* sp.)) (Table 23). The higher organic carbon content might be due to higher organic manure addition through farm yard manure which is in agreement with earlier report by Ferinni *et al.* (2008). The decrease in the organic carbon at harvest might be due to the uptake of nutrients by the plants.

The initial available nitrogen content also decreased after the experiment. The nitrogen might have been taken up by the plant. Among the treatments, *Bacillus* sp. (T_4) recorded the highest available nitrogen content (168 mg kg^{-1}) which was on par with *Trichodema* sp. (T_5). It has been reported that some plant growth promoting bacteria secrete some molecules which acts as an inducing signals to help the process of nitrogen fixation (Sharma *et al.*, 2007). The initial available phosphorus content was 72.70 mg kg^{-1} and increased at the end of the experiment in all the treatment. The highest available phosphorus in the potting mixture was recorded in TMT (*Trichodema* sp.) + BML (*Bacillus* sp.) (T_3) with 775 mg kg^{-1}. Phosphorus solubilizing microorganisms can transform the insoluble P to soluble P very slowly during solubilisation process (Chang and Yang, 2009). Similarly, He *et al.* (2002) also reported that inorganic form of P is solubilized by microorganisms excreting organic acids that dissolve phosphatic minerals and release P into the soil solution. This might have led to the availability of easily available P for the plants (Srilatha and Harish Kumar, 2015) which is in agreement with the present study. In the case

of available potassium, the potassium content decreased at the end of the experiment. However, highest available potassium was recorded in the case of PGPR Mix- II (T_9) with 634.5 mg kg^{-1}. Subbiah (1990) reported that when adequate amount of farm yard manure was added to soil with biofertilizer, it improved biofertilizer efficiency and ultimately nutrient status of the soil. In the present studies, the FYM was used in the potting mixture which might have increased the availability of potassium.

5.8. Population of *Trichoderma* sp. and *Bacillus* sp. in the potting mixture at the time of flowering and harvest

In general, population of inoculated microorganisms decline more/less rapidly due to introduction into a natural soil which is microbiologically undisturbed soil (Bashan *et al.,* 1995). In the present study, the inoculated biofilm based inoculants in the potting mixture revealed that the population of *Trichoderma* sp. declined from 10^7 to 10^2 cfu g^{-1} of potting mixture at the time of harvest (Table 31). However, the highest *Trichoderma* sp. was recorded in the case of biofilm based inoculant TPZ (*Trichoderma* sp.) + BPN (*Bacillus* sp.) (T_1) followed by *Trichoderma* sp. (T_5) (3.1 x 10^2 cfu g^{-1}). Among all the treatments, biofilm based inoculants performed better than the other treatments. The decline in the population might be due to abiotic stress factors such as soil texture, pH, temperature, moisture content and substrate availability which largely determine the survival and activity of introduced microorganisms (Gray, 1975), for which a suitable formulation for biofilm based inoculants needs to be developed. In the present studies, the biofilm based inoculants performed better with respect to the population of *Trichoderma* sp. Donlan (2002) reported that microbial species associated with surface and enclosed in extra cellular polymeric matrix provides enhanced survival ability to the species under adverse environmental conditions which is in agreement with the present study where the population of *Trichoderma* sp. was better in biofilm based inoculants.

In the case of *Bacillus* sp., population before and after the experiment revealed that the population declined from 10^8 to 10^2 cfu g^{-1} (Table 32). However, the population of biofilm based inoculants were higher than the other treatments. It has been reported that biofilm formulations provide protection against environment

stresses, antimicrobial compounds and acquisition of new genetic traits (Stewart, 2002; Rafique *et al.*, 2015) which is in agreement with the present results.

The present studies indicated that *Trichoderma* based *Bacillus* sp. biofilms could be a promising inoculant for the growth promotion and biocontrol of plant pathogens due to the dual attributes of the inoculants. Among all the treatments, biofilm based *Trichoderma* sp. –TCH (Chellakara)- *Bacillus* sp.-BCH (Chellakkara) inoculant (T_2) was the most promising inoculant for the management of diseases and growth promotion in cowpea. The present study suggests that combining PGP traits with plant growth promotion and antagonistic activity against phytopathogens are more effective than single inoculants. However, further studies are needed to confirm the results under field conditions and research is needed to increase the shelf life of biofilm based inoculant.

5. 9. Future line of work

- Species level identification of three most promising isolates of *Trichoderma* sp. (TCH, TPZ and TML).
- Development of formulation for enhancing the shelf life of biofilm based inoculants
- Field evaluation of the most promising biofilm based inoculant for disease management and growth promotion in cowpea
- Evaluation of biofilm based inoculants for other crops and diseases
- In the case of *Pythium*, the disease management experiment may be done during rainy cool period

6. SUMMARY

The present study on "Evaluation of biofilm based microbial antagonists for the management of soil borne diseases and growth promotion in cowpea (*Vigna unguiculata* L. Walp)" was carried out in the Department of Agricultural Microbiology, College of Horticulture, Vellanikkara during 2015-2017. The major objectives were to increase the survivability of *Trichoderma* sp. and *Bacillus* sp. through biofilm production and to evaluate the biofilm based biocontrol agents for the management of rot diseases and growth promotion in cowpea under pot culture studies. The important findings of the study are summarized below:

- Nine *Trichoderma* sp. and five *Bacillus* sp. were obtained from ten rhizosphere soils of cowpea growing areas of Thrissur district. The highest population of *Trichoderma* sp. was recorded in Chellakara location (4.8 x 10^3 cfu g^{-1}) and the highest *Bacillus* sp. population was recorded in Pananchery location (4.48 x 10^3 cfu g^{-1}).
- Among *Trichoderma* sp., maximum IAA production was recorded by TCK-2 from Chalakudy (24.03 µg ml^{-1}) followed by TML from Mala (14.77 µg ml^{-1}). Among *Bacillus* sp., maximum IAA production (6.20 µg ml^{-1}) was recorded by BCH isolate from Chellakara location.
- Among *Trichoderma* sp., four isolates (TPZ (Pazhayanur), TCH-1 (Chellakara), TMS (Mullassery) and TMT (Mattathur)) were positive for Phosphate solubilisation. The maximum P solubilisation was recorded by TPZ from Pazhayanur (147 µg ml^{-1}) followed by TMT from Mattathur (139.6 µg ml^{-1}). Among *Bacillus* sp., maximum P solubilisation was recorded by BPN isolate from Pananchery (151.3 µg ml^{-1}) followed by BML from Mala (139.6 µg ml^{-1}).
- Among *Trichoderma* sp., two isolates (TCH-1 from chellakara and TPZ from Pazhayanur) were positive for HCN production, five isolates (TCH-1 from Chellakara, TMT from Mattathur, TMS from Mullassery, TPZ from Pazhayanur and TCK-1 from Chalakudy) were positive for ammonia production. Among *Bacillus* sp., two isolates (BCH from Chellakara and BPN from Pananchery) were positive

for HCN production. Two isolates (BML from Mala and BPN from Pazhayanur) were positive for siderophore production.

- *Trichoderma* sp. and *Bacillus* sp. were screened *in vitro* for their antagonistic activity against *Rhizoctonia solani* and *Pythium aphanidermatum*. Among *Trichoderma* sp., TCH (Chellakara) isolate recorded maximum inhibition (51.1 %) against *Rhizoctonia solani* and *Pythium aphanidermatum* (57.7 %). None of the *Bacillus* sp. showed antagonistic activity against *Rhizoctonia solani* and *Pythium aphanidermatum*.

- *Bacillus* sp. were screened for biofilm production using three different methods viz., tube method, congo red agar medium method and tissue culture plate method. Based on all the methods, BCH (Chellakara) isolate (0.0600) was the most promising biofilm producer followed by BPN (Pananchery) (0.058) and BML (Mala) (0.056).

- Based on the plant growth promoting traits, antagonistic activities, three most efficient *Trichoderma* sp. isolates (TCH, TPZ and TMT) were selected. Similarly, based on the plant growth promoting traits, antagonistic and biofilm production, three most efficient *Bacillus* sp. (BCH, BPN and BML) isolates were selected for further studies.

- Three best *Trichoderma* based *Bacillus* sp. biofilms were selected viz., TPZ+BPN, TCH+BCH and TMT+BML based on the growth promotion, antagonistic activity, biofilm production and compatibility under *in vitro*.

- These *Trichoderma* based *Bacillus* sp. biofilms were evaluated for their efficiency in growth promotion and disease management in cowpea in comparison with treatment involving individual isolates of *Trichoderma* sp. and *Bacillus* sp., *Trichoderma* sp. + *Bacillus* sp., Carbendazim+Mancozeb, POP recommendation (KAU, 2011), PGPR Mix-II.

- The soil pH and nutrient status of the potting mixture before the experiment showed 7.09 pH, 3.05 % organic carbon, 0.47d Sm^{-1}, 373.74 mg kg^{-1} available nitrogen, 72.40 mg kg^{-1} available phosphorus, 917.09 mg kg^{-1} available potassium.

- With the challenge inoculation of *Rhizoctonia solani*, the maximum germination per cent (42.8 %) was recorded in TCH (*Trichoderma* sp.) + BCH (*Bacillus* sp.) (T_2) which was on par with PGPR Mix- II (T_9). The highest cowpea yield (52.28 g $plant^{-1}$) was recorded in T_9 (PGPR Mix- II). Among biofilm based treatments TCH (*Trichoderma* sp.) + BCH (*Bacillus* sp.) (T_2) recorded maximum yield (48 g $plant^{-1}$) after challenge inoculation with *Rhizoctonia solani.*

- Under challenge inoculation of *Pythium aphanidermatum*, higher cowpea yield (51.71 g $plant^{-1}$) was recorded in PGPR Mix- II (T_9). Among biofilm based treatments, TCH (*Trichoderma* sp.) + BCH (*Bacillus* sp.) (T_2) recorded maximum yield (47.42 g $plant^{-1}$).

- Treatments T_2 (TCH+BCH) and T_9 (PGPR Mix- II), recorded zero per cent collar rot disease incidence while T_6 (*Bacillus* sp.)+(*Trichoderma* sp.) and T_8 (POP KAU,2011) recorded highest (42.85) per cent disease incidence up to 70 DAS.

- No root rot disease incidence was observed throughout the experimental period.

- Based on the biometric parameters, per cent collar rot disease incidence and yield parameters, biofilm based formulation TCH (*Trichoderma* sp.)+ BCH (*Bacillus* sp.) (T_2) was the most promising treatment for the management of collar rot disease and growth promotion in cowpea, which was on par with the PGPR Mix- II (T_9).

- Based on biometric parameters, per cent root rot disease incidence and yield parameters, biofilm based formulation of TCH (*Trichoderma* sp.) + BCH (*Bacillus* sp.) (T_2) was the best treatment for the management of root rot disease and growth promotion in cowpea, which was on par with the PGPR mix- II (T_9).

- Population of inoculated *Trichoderma* sp., *Bacillus* sp. and *Rhizobium* sp. in the potting mixture showed declining trend till the final harvest of the crop. The population decreased from 10^8 cfu ml^{-1} to 10^4 cfu ml^{-1}. However, biofilm based inoculant showed highest population of *Trichoderma* sp. and *Bacillus* sp. at the time of harvest.

- 16S rRNA sequence analysis was carried out for the identification of three most promising biofilm producing *Bacillus* sp. isolates. The BCH isolate from

Chellakara showed homology (92 %) with *Bacillus subtilis*, BML from Mala showed homology (91 %) with *Bacillus megaterium* and BPN from Pananchery showed homology (91 %) with *Bacillus venezensis*.

- Based on all the parameters, TCH (*Trichoderma* sp.) + BCH (*Bacillus* sp.) (T_2) treatment was the best among biofilm based inoculant for disease management and growth promotion in cowpea.

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EVALUATION OF BIOFILM BASED MICROBIAL ANTAGONISTS FOR THE MANAGEMENT OF SOIL BORNE DISEASES AND GROWTH PROMOTION IN COWPEA (*Vigna unguiculata* L. Walp)

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ABSTRACT OF THE THESIS

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Abstract

The biocontrol agents play an important role in the plant disease management and growth promotion. They are eco-friendly and low cost agricultural inputs. One of the major constraints in the use of biocontrol agents, is the survivability of inoculated cultures till the end of the crop period. So, there is a need to enhance the survivability of the biocontrol agents for the management of soil borne pathogens and growth promotion. *Trichoderma* and *Bacillus* sp. are two well-known biocontrol agents for plant disease management and growth promotion. Cowpea is one of the most popular, protein rich legume crop of Kerala. One of the major constraints in the cowpea production is the diseases like collar-rot (*Rhizoctonia solani*) and root rot (*Pythium aphanidermatum*) which have become a serious threat. Since, no studies have been conducted in Kerala on the biofilm based inoculants, an attempt was made to increase the survivability of *Trichoderma* and *Bacillus* through biofilm based inoculants for the management of two major soil borne diseases and growth promotion in cowpea.

Rhizosphere soil samples were collected from ten different cowpea growing areas of Thrissur district. The maximum population of *Trichoderma* sp. (4.8×10^3 cfu g^{-1}) and *Bacillus* sp. (4.48×10^5 cfu g^{-1}) were recorded in Chellakara and Mala, respectively.

A total of nine *Trichoderma* sp. and five *Bacillus* sp. were obtained. They were screened for plant growth promoting and antagonistic activities. Among *Trichoderma* sp., maximum IAA production (24.03 μg ml^{-1}) was recorded by TCK-2 (Chalakudy) followed by TML (Mala) (14.77 μg ml^{-1}). TML (Mala) isolate was the most efficient P solubilizer (147.2 μg/ml). Among *Bacillus* sp., maximum IAA production (6.20 μg ml^{-1}) and maximum P solubilization (151.3 μg ml^{-1}) were recorded by BCH (Chellakara) isolate.

Among *Trichoderma* sp., TCH-1 (Chellakara) and TMT (Mttathur) were positive for HCN production and TCH-1, TMT, TMS, TPZ and TCK-1 were positive for ammonia production. Among *Bacillus* sp., BCH (Chellakara) and BMT (Mattathur) were positive for HCN production and all five isolates (BCH, BML, BMS, BPN and BMT) were positive for ammonia production.

Both *Trichoderma* sp. and *Bacillus* sp. were screened *in vitro* for their antagonistic activity against *Rhizoctonia solani* and *Pythium aphanidermatum*. Among *Trichoderma* sp., TCH-1 (Chellakara) isolate recorded maximum inhibition (51.1%) against *Rhizoctonia solani* and *Pythium aphanidermatum* (57.7%).

Bacillus sp. isolates were screened for biofilm production and highest biofilm production was recorded by BCH (Chellakara) (0.060) followed by BPN (Pazhayanur) (0.058) isolates.

Based on the PGPR traits, per cent inhibition and biofilm production under *in vitro*, three most promising *Trichoderma* sp. (TCH, TMT, TPZ) and *Bacillus* sp. (BCH, BPN, BML) were subjected for mutual compatibility studies. All the selected *Trichoderma* sp. and *Bacillus* sp. were mutually compatible with each other.

The isolates of TCH (Chellakara) + BCH (Chellakara), TPZ (Pazhayanur) + BPN (Pazhayanur) and TMT (Mattathur) + BML (Mala) were selected for the biofilm based inoculant production. Among the different carrier materials for biofilm based inoculant production talc powder was the most promising carrier material due to highest population of isolates at 90 DAI.

Biofilm based inoculants were evaluated under pot culture for the management of collar rot (*Rhizoctonia solani*) and root rot (*Pythium aphanidermatum*) in cowpea under two separate experiments. Based on the biometric parameters, collar rot and root rot disease management and yield parameters, biofilm based formulation of TCH (Chellakara)+BCH (Chellakara) (T_2) was the most promising treatment for the management of collar rot disease and growth promotion in cowpea, which was on par with the PGPR Mix- II (T_9).

Population of inoculated *Trichoderma* sp., *Bacillus* sp. and *Rhizobium* sp. indicated declining trend till the final harvest of the crop. The population decreased from 10^8 cfu ml^{-1} to 10^4 cfu ml^{-1}. However, biofilm based inoculant showed highest population of *Trichoderma* sp. and *Bacillus* sp. at the time of harvest.

The three best promising *Bacillus* sp. isolates were identified through 16S rRNA sequencing and identified BCH isolate as *Bacillus subtilis*, BPN as *Bacillus velezensis* and BML as *Bacillus megaterium*.

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