GROWTH PROMOTION IN CHILLI (*Capsicum annuum* L.) ON INOCULATION WITH *Pseudomonas fluorescens* AND *Piriformospora indica*

by

NANDANA M.S.

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled "Growth promotion in chilli (*Capsicum annuum* L.) on inoculation with *Pseudomonas fluorescens* and *Piriformospora indica*" 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.

Vellayani,
Date:

Nandaana M. S.
(2017-11-078)

CERTIFICATE

Certified that this thesis entitled "**GROWTH PROMOTION IN CHILLI (*Capsicum annuum* L.) ON INOCULATION WITH *Pseudomonas fluorescens* AND *Piriformospora indica*"** is a record of research work done independently by **Ms. NANDANA M. S. (2017-11-078)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

Vellayani,
Date:

Dr. K. N. Anith
(Major Advisor, Advisory Committee)
Professor
Department of Agricultural Microbiology
College of Agriculture, Vellayani
Thiruvananthapuram - 695522

CERTIFICATE

We, the undersigned members of the advisory committee of Ms. Nandana M. S. (2017-11-078) a candidate for the degree of Master of Sciences in Agriculture with major in Agricultural Microbiology, agree that the thesis entitled ""**GROWTH PROMOTION IN CHILLI (*Capsicum annuum* L.) ON INOCULATION WITH *Pseudomonas fluorescens* AND *Piriformospora indica*"** may be submitted by Ms. Nandana M. S. in partial fulfilment of the requirement for the degree.

Dr. K. N. Anith
(Major Advisor, Advisory Committee)
Professor
Department of Agricultural Microbiology
College of Agriculture, Vellayani
Thiruvananthapuram - 695522

Dr. K.S. Meenakumari,
Professor (Plant Pathology) and Head
Department of Agricultural Microbiology
College of Agriculture,
Vellayani.

Dr. N.V. Radhakrishnan,
Professor (Plant Pathology) and Head
Coconut Reseach Station
Balaramapuram
Thiruvananthapuram.

Dr. S. Sarada,
Assistant Professor (Horticulture)
Department of Vegetable Sciences
College of Agriculture
Vellayani.

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"Growth is never by mere chance; it is the result of forces working together"

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS AND SYMBOLS USED

et. al.	And other co-workers
ACW	Autoclaved coconut water
cfu	Colony forming unit
cm	Centimetre
CRD	Completely randomized design
CD	Critical difference
CWA	Coconut water agar
DAI	Days after inoculation
DAT	Days after transplanting
^{0}C	Degree celsius
DNA	Deoxyribo nucleic acid
Fig.	Figure
FAO	Food and Agricultural Organisation
g	Gram
h	Hours
ha	Hectare
KB	King's B
m	Metre
µg	Microgram
µL	Microliter
mg	Milligram
viz.,	Namely
nm	Nanomertre
NS	Non-significant
No.	Number
%	Per cent
PGPR	Plant growth promoting rhizobacteria
rpm	Rotations per minute

sp. or spp.	Species (singular and plural)
SE (m)	Standerd error (Mean)
var.	Variety

LIST OF APPENDICES

Sl. No.	Title	Appendix No.
1.	Composition of media used	

Introduction

INTRODUCTION

Chilli, known as wonder spice is one among the most important commercial spice crop around the world and is best known for its hot, pungent flavor. It is an excellent source of Vitamin, A, B, C and E with minerals like molybdenum, manganese, folate, potassium, thiamin, and copper. It contains various unique plant compounds like capsaicin, which gives pungency and pigment capsanthin which gives red colour.

Different varieties of chilli are cultivated for varied uses like vegetable, pickles, spice and condiments. It is mainly raised by seedling using plug trays (pro-trays). This system helps the grower for establishing seedlings with perfect stands, uniform physiological plant age and optimal spacing during transplanting and thus enable quicker re-establishment and less transplanting shock. The use of healthy seedlings with well-established root system helps to avoid transplantation shock, when the seedlings are transferred to the field from nursery. The use of biological agents at the nursery production stage is advantageous as many of them enhance better rooting and health of the seedlings.

Application of chemicals in the form of pesticides and fertilizers has caused incurable injury to the overall ecosystem. Scientists working on alternatives have recognized the potential of microbes which can help in increasing the yield, and can also be used as biocontrol agents, without causing the damage associated with chemical fertilizers (Nakkeeran *et al*., 2005). The use of plant growth promoting rhizobacteria (PGPR) is an environmentally sound way of enhancing crop yields by improving plant growth through either direct or indirect mechanisms. The mechanisms of PGPR include solubilizing nutrients for easy uptake by plants, regulating nutritional and hormonal balance and inducing resistance against plant pathogens by synergistic and antagonistic interactions.

Pseudomonas fluorescens, a common Gram negative, rod-shaped bacterium is a member of plant growth promoting rhizobacteria (PGPR) (Palleroni, 1984). It rigorously colonize roots and provide beneficial effects to the plant development. It enhances plant growth by producing a variety of useful metabolites such as phytohormones like Indole-3-acetic acid, siderophores, antifungal compounds like 2,4-diacetyl phloroglucinol, antibiotic compound like pyrollnitrin, and solubilize phosphorus and important macro elements for the growth of the plants (Sivasakthi *et al.*, 2014). Certain members of the *P. fluorescens* have been shown to be potential agents for the biocontrol which suppress plant diseases by protecting the plants from fungal infection. *P. fluorescens* has simple nutritional requirements and grows well in mineral salts medium supplemented with a carbon source. As they are well adapted in soil, *P. fluorescens* strains are being exploited extensively for biocontrol of pathogens in agriculture and bioremediation of various organic compounds.

Several reports suggest that root colonizing bio-agents also colonize the internal tissues of the plant roots thus making them to be considered as endophytes. Endophytes are microorganisms that asymptomatically grow within the plant tissues without causing any disease or gaining benefit other than residency. They include bacteria and fungi that can be isolated from surface-disinfected plant tissue or extracted from inside the plant which does not cause any visible harm (Hallmann *et al.*, 1997). Endophytic fungi live in symbiotic association with plants and play a major role in plant's resistance to various biotic, abiotic stresses and diseases, higher seed yield and plant growth promotion. Many are able to produce plant growth hormones, antimicrobial compounds and various bioactive metabolites. These mycoendophytes have the potential for getting developed into economically and eco-friendly viable agricultural products.

Piriformospora indica is a wide host range root colonizing endophytic fungus which allows the plant to grow under extreme physical and nutrient stress condition. The fungus can be cultivated on complex or mineral substrate. It belongs to the

Sebacinales in Basidiomycota (Verma *et al.*, 1998; Weiss *et al.*, 2004; Franken, 2012; Varma *et al*, 2012). This endophytic fungus has a wide host range including mono and dicot plants, gymnosperms, pteridophytes and bryophytes. It grows inter- and intracellularly, forming pear shaped chlamydospores in root cortex.

Root colonization of *P.indica* has resulted in increased nutrient uptake, temperature and salt stresses, and confers systemic resistance to pathogenic organisms, insects, toxins and heavy metals. It enhances biomass production, stimulate early flowering and seed production. It is used as a potential microorganism for biological hardening in tissue culture raised plants (Verma *et al.*, 1998; Yadav *et al.*, 2010; Das *et al.*, 2012).

Piriformospora indica is usually grown on modified Hill–Kafer synthetic medium and potato dextrose agar medium (Pham *et al.,* 2004). *P. indica* grow well in solid coconut water agar and autoclaved coconut water (Anith, 2009). Coconut water is a cheap and readily obtainable material for microbial cultivation. It is naturally free from microbial contamination as it is preserved inside the shells of coconut. It is a highly nutritious medium rich in vitamins, minerals and amino acids. The bioactivity of coconut water is due to the various chemical compounds which are essential to biochemical, biotechnology and agriculture field. Various plant hormones like auxin, cytokinin, gibberlic acid and indole -3-acetic acid are also present in coconut water (Jean *et al.*, 2009) in which cytokinin present in the form of kinetin and trans zeatin are gaining more importance now. Coconut water, being rich in nutrients can be used for multiplication of micro-organisms like bacteria (Unagul *et al.*, 2007) and fungi (Anith, 2009). Multiplication of PGPR in coconut water is an easy method for farmers during nursery production of vegetative propagules.

The interaction of *P. indica* with various rhizobacteria has been studied. The fungus interact with various rhizobacteria in different manner. Growth and root colonization of *P. indica* is promoted by rhizobacteria like *Pseudomonas putida* while

some other bacteria behave neutral or inhibit the growth of the fungus (Varma *et al*, 2012).

Piriformospora indica can be co-cultured with various bacteria. The consistency and level of growth promotion and pest control achievable with these beneficial organisms varies, when they are grown together in a co-culture and grown separately in pure culture and applied individually or as mixed inoculation (Anith *et al*., 2015). Sometimes the enzyme activity of various PGPR can be enhanced by co-culturing it with fungus and the level of secretion of various metabolies by bacteria and the acidity of the medium is also varied in the co-culturing system (El-katanty *et al*., 2010). Fungal bacterial interaction and biofilm formation enhances the effect of biodegradation compared to monoculture (Seneviratne *et al.,* 2006).

A consortium of beneficial microorganisms improves the efficiency of a bioinoculant formulation by improving the consistency of its performance (Vidhyasekaran and Muthamilan 1995; Nakkeeran *et al.,* 2005). Developing a plant growth promoting and biological control system by formulating a microbial consortium need a detailed study of *in vitro* and *in vivo* interaction of these bioagents. A microbial consortium having efficient root endophytic colonizers along with a PGPR will be of great practical significance as an effective bioinoculant especially in the nursery production.

Endophytes applied alone or as a consortium with PGPR is gaining importance nowadays due to its excellent potential as plant growth promotor and biocontrol agent. *P. indica*, an axenically cultivable root endophytic fungi along with mutualistic rhizobacteria act as a "biological trigger" to enhance the plant growth and activate the stress response. Co-culturing of these two organisms in cheaply available medium like coconut water will make the biofertilizer production a farmer friendly, affordable and effective method. The present study was undertaken considering these key points, with an objective of assessing the compatibility of *P. indica* along with two *Pseudomonas fluorescens* strain and evaluate their effect on growth promotion in chilli.

Review of Literature

2. REVIEW OF LITERATURE

Chilli, (*Capsicum annuum* L.), also called red pepper, belongs to the genus capsicum, under the solanaceae family. It is believed to have originated in South America. Chillies are integral and the most important ingredient in many different cuisines around the world as it adds pungency, taste, flavour and color to the dishes. Indian chilli is considered to be world famous for two important commercial qualities; its colour and pungency levels. Some varieties are famous for the red colour because of the pigment capsanthin and others are known for biting pungency attributed to capsaicin. The other quality parameters in chilli are length, width and skin thickness (Shaheen *et al.,* 2018)

India is the largest cultivator and consumer of chilli. India is meeting approximately 25% of the world's chilli requirement and considered to be leader in chilli export followed by China with a share of 24%. Indian chilli exports are mainly influenced by domestic demand and uneven production which is interrupted by erratic monsoon, drought, and yield factor. Indian chilli exports are facing problems of quality in terms of right pungency, yield and colour value as well as pesticide residues and aflatoxin. Both domestic consumption and export of chilli necessitates production of quality chillies devoid by contamination of pesticides, industrial chemicals and aflatoxins (Bera *et al.,* 2018).

A large amount of herbicides, pesticides, and fertilizers is applied every year to achieve maximum productivity of chilli and to meet the growing demand, the use of chemical fertilizers in India has increased 170 times in last 50 years (FAO, 2010). This is a major environmental and health concern considering the deleterious impact of these chemical compounds on terrestrial and aquatic ecosystems.

Biological control encompasses the use of biological organisms, especially microorganisms including bacteria and fungi in disease or pest management (Yuliar

et al., 2015). There are many rhizosphere inhabiting microbial agents like fluorescent *Pseudomonas* and *Bacillus* spp. that are effective in deterring soil borne plant diseases in several crops. Most of these biological control agents also possess plant growth promoting abilities, therefore referred to as Plant Growth Promoting Rhizobacteria (PGPR). PGPR constitute approximately 2-5% of the total rhizomicrobial population (Kloepper *et al.*, 1980; Antoun *et al.*, 1998). There were several reports of isolation and characterization of PGPR and phosphate solubilizing bacteria from chilli rhizosphere (Ponmurugan and Gopi, 2006; Datta *et al.,* 2011; Kumar and Audipudi, 2015; Gowtham *et al.,* 2018).

2.1. PLANT GROWTH PROMOTING RHIZOBACTERIA

Kloepper and Schroth (1981) coined the term PGPR for the first time to illustrate the population of beneficial microbes that shows the plants growth promotion by effectively colonizing the plant roots. Several significant interactions among plants and beneficial organisms occur in rhizospheric soil. Within rhizospheric soil, among all the coexisted microorganisms, bacteria are in high proportion. By colonizing plant roots, bacteria releases certain enzymes and exudates that protect plant from the pathogenic microorganisms existing in the soil (De Garcia-Salamone *et al.*, 2006).

Plant growth promoting rhizobacteria can be classified into extracellular plant growth promoting rhizobacteria (ePGPR) and intracellular plant growth promoting rhizobacteria (iPGPR) (Viveros *et al.*, 2010). The ePGPR may exist in the rhizosphere, on the rhizoplane or in the spaces between the cells of root cortex while iPGPR locate generally inside the specialized nodular structures of root cells. The bacterial genera such as *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcous, Pseudomonas* and *Serratia* belongs to ePGPR (Ahemad *et al.*, 2014). The iPGPR belongs to the family of Rhizobiaceae that includes *Allorhizobium, Bradyrhizobium, Mesorhizobium* and *Rhizobium,* endophytes and *Frankia* species both

of which can symbiotically fix atmospheric nitrogen with higher plants (Bhattacharyya and Jha, 2012). PGPR directly or indirectly provide protection to the plants.

Most of the PGPR isolates rapidly colonize the rhizospheric soil and enhance the nutrient uptake ability of plants by solubilizing the nutrients into absorbable form. Many of them fix atmospheric nitrogen and are termed as biological nitrogen fixing bacteria (BNF) and it is documented that BNF contributes about 180×10^6 metric tons nitrogen per year across the globe (Cupples, 2005). Phosphate solubilizing bacteria (PSB) yet another PGPR group that includes strains of *Pseudomonas* sp. and *Bacillus* sp. The use various mechanisms such as release of certain enzymes and acid production to solubilize unavailable phosphate (Greiner *et al.,* 2001). PGPR produce siderophores to compete and attain Fe^{3+} (ferric ions) from surrounding under iron scarcity (Whipps, 2001).

PGPR hold significant importance to enhance the plant's growth parameters by producing phytohormones. *P. putida* and *P. fluorescens* are the two most significant strains of *Pseudomonas* that presented significant outcomes in auxin production and growth promotion of crop plants (Khakipour *et al*., 2008). Iqbal *et al.,* (2012) reported improved nodule number, nodule dry weight, fresh biomass, grain yield, straw yield, and nitrogen content in grains of lentil as a result of lowering of the ethylene production via inoculation with plant growth promoting strains of *Pseudomonas* sp containing ACC deaminase along with *R. leguminosarum*.

The efficiency of PGPR as biocontrol agents is determined by variety of indirect mechanisms like production of antibiotics, siderophores, HCN, hydrolytic enzymes etc. (Viveros *et al.,* 2010). Antibiosis, the production of antibiotics is considered to be one of the most powerful and well studied biocontrol mechanisms of plant growth promoting rhizobacteria against phytopathogens. Bacterization of wheat seeds with *P. fluorescens* strains producing the antibiotic phenazine-1- carboxylic acid

(PCA) resulted in significant suppression of take-all disease in about 60% of field trials (Weller, 2007).

Growth enhancement through enzymatic activity is another mechanism used by plant growth promoting rhizobacteria. PGPR strains can produce certain enzymes such as chitinases, dehydrogenase, β-glucanase, lipases, phosphatases, proteases etc. (Joshi *et al.*, 2012). *Pseudomonas fluorescens* CHA0 suppresses black root rot of tobacco caused by the fungus *Thielaviopsis basicola* was attributed to its ability to produce various lytic enzymes (Voisard *et al.*, 1989).

Biopriming plants with some plant growth promoting rhizobacteria can also provide systemic resistance against a broad spectrum of plant pathogens. Induced systemic resistance involves jasmonate and ethylene signaling within the plant and these hormones stimulate the host plant's defense responses against a variety of plant pathogens (Glick, 2012).

2.1.1. PLANT GROWTH PROMOTING RHIZOBACTERIA AND CHILLI

The major constrains in chilli cultivation are the pest and disease problem and lack of healthy seedlings production in nursery. The use of plant growth promoting rhizobacteria (PGPR) in seedling stage in nursery is a better alternative to solve this problem. They play an important role to increase in soil fertility, plant growth promotion, and suppression of phytopathogens for development of ecofriendly sustainable agriculture.

A study conducted on the application of 15 rhizospheric isolates to chilli resulted in remarkable increase in growth characteristics such as total number of fruits, fruit-weight, and yield in field conditions (Datta *et al.,* 2011).

It was reported that bacterial strains isolated from chilli rhizosphere, when screened for plant growth promoting traits such as indole acetic acid production, phosphate solubilization, hydrogen cyanide production, siderophore production and

ammonia production revealed that one of the isolates *Stenotrophomonas maltophilia* AVP 27, is a promising plant growth promoting rhizobacteria with wide variety of mechanisms (Kumar and Audipudi, 2011)

Gowtham *et al.,* (2018) documented that chilli seed treatment with *Bacillus amyloliquefaciens,* a PGPR isolated from chilli rhizosphere resulted in maximum enhancement of seed germination, seedling vigour along with an increase in vegetative growth parameters.

A study conducted on the effect of the bacteria *Pseudomonas fluorescens* AMB- 8 grown in coconut water with additives in chilli disclosed that the bacterial strain enhanced the growth of chilli seedlings (Anith *et al.*, 2016).

2.1.2 PSEUDOMONAS FLUORESCENS, A POTENTIAL PGPR

Fluorescent Pseudomonads are considered to be the most promising group of plant growth promoting rhizobacteria involved in biocontrol of plant diseases. They produce secondary metabolites such as antibiotics, phytohormones, volatile compound hydrogen cyanide and siderophores (Lata *et al.,* 2002).

Pseudomonas possesses many traits that make them well suited as biocontrol and growth promoting agents. These include the ability to (i) grow rapidly *in vitro* and to be mass produced; (ii) efficiently utilize seed and root exudates; (iii) colonize and multiply in the rhizosphere and in the interior of the plant; (iv) produce a wide spectrum of bioactive metabolites (that is, antibiotics, siderophores, volatiles and growth promoting substances); (v) compete aggressively with other microorganisms; and (vi) adapt to environmental stresses (Sivasakthi *et al.*, 2014).

Various studies revealed that this PGPR is able to substitute the use of chemical fertilizer to a greater extent. In a study conducted on banana plants *Pseudomonas fluorescens* promoted banana growth similarly or even slightly superior to 100%

chemical fertilization (Gamez *et al.*, 2019). Plant growth promoting ability of these bacteria is mainly because of the production of indole -3- acetic acid, siderophores and antibiotics (Sivasakthi *et al.*, 2014).

Karnwal (2009) obtained 30 fluorescent *Pseudomonas* isolates from different plant rhizosphere and characterized them on the basis of biochemical tests and plant growth promoting activities. *P. fluorescens* and *P. aeruginosa* showed the best plant growth promoting activity in rice paints. For both strains, indole production increased with increases in tryptophan concentration in the growth medium.

Saranraj *et al.* (2013) collected the paddy rhizosphere soil sample from ten different locations in Tamil Nadu. Isolates obtained from the samples were tested for their efficiency of IAA and siderophore production. The maximum IAA production and siderophore production was recorded by isolate PF-8, a *P. fluorescens* strain and the rice plants treated with this isolate showed enhanced growth compared to the other plants.

Presence of various potential plant growth-promoting properties including indole acetic acid and siderophore production was reported in *Pseudomonas aeruginosa.* The growth enhancement effect of this *Pseudomonas aeruginosa* isolates on chilli showed promising results, and the growth parameters were found to be statistically significant when compared to control (Linu *et al.,* 2019).

Phosphorus is major essential macronutrients for biological growth and development. Microorganisms offer a biological rescue system capable of solubilizing the insoluble inorganic P of soil and make it available to the plants. The ability of some microorganisms to convert insoluble phosphorus to an accessible form, like orthophosphate, is an important trait in a PGPB for increasing plant yields (Rodriguez and Fraga, 1999).

About 752 fluorescent pseudomonad isolates were recovered from the rhizospheres of wheat and barley by Browne *et al.,* in 2009 and classified them as strong, weak or non-phosphate solubilizers on the basis of clearing zones formed on medium containing insoluble $Ca_3(PO_4)_2$. 82% of the strong-solubilizing isolates were clustered into the taxonomic group *P. fluorescens* having an enhanced capacity to mobilize inorganic phosphate in agricultural soils.

In a study conducted by Mehnas *et al.* (2009) it was a reported about the isolation, identification, and characterization of seven fluorescent pseudomonads from the roots, shoots, and rhizosphere soil of sugarcane and their impacts on the growth of sugarcane plantlets and it was observed that all seven isolates provided significant increases in fresh and dry masses and five strains increased shoot height.

It was reported that bio-priming with *Pseudomonas putida* strain BA 8 under saline conditions could be useful to obtain higher seed germination percentages in radish (Kaymak, 2009).

Egamberdieva (2010) analyzed plant growth promoting bacteria for their growth-stimulating effects on two wheat cultivars in pot experiments using calcareous soil. The results showed that bacterial strains *P. fluorescens* NUU2 was able to colonize the rhizosphere of both wheat cultivars and significantly stimulate the shoot and root length and dry weight of wheat.

Siderophore production confers competitive advantages to PGPR that can colonize rhizosphere and exclude other microorganisms from this ecological niche (Haas and Défago, 2005). Among most of the bacterial siderophores studied, those produced by pseudomonads are known for their high affinity to the ferric ion. The potent sideophore, pyoverdin, for example, can inhibit the growth of bacteria and fungi that produce less potent siderophores in iron-depleted media *in vitro* (Kloepper *et al.* 1980).

A pseudobactin siderophore produced by *P. putida* B10 strain was able to suppress *Fusarium oxysporum* in soil deficient in iron and this suppression was lost when the soil was replenished with iron, a condition that represses the production of iron chelators by microorganisms (Kloepper *et al.*, 1980).

Besides siderophore production, the biocontrol abilities of pseudomonad strains essentially depend on aggressive root colonization, induction of systemic resistance in the plant, and production of antifungal antibiotics (Haas and Keel, 2003). The production of one or more antibiotics is the mechanism most commonly associated with the ability of plant growth-promoting bacteria to act as antagonistic agents against phytopathogens (Glick *et al.*, 2007).

Pyrrolnitrin, the antibiotic produced by the *P. fluorescens* BL915 strain is able to prevent the damage of *Rhizoctonia solani* during damping-off of cotton plants (Hill *et al.*, 1994).

Non-pathogenic rhizobacteria have been shown to suppress disease by inducing a resistance mechanism in the plant called "Induced Systemic Resistance" (ISR) (Van Loon *et al.*, 1998). ISR was formerly described by Van Peer *et al.* (1991) in carnation plants that was systemically protected by the *P. fluorescens* strain WCS417r against *F. oxysporum* f. sp. *dianthi*.

Rhizospheric bacterial communities have efficient systems for uptake and catabolism of organic compounds present in root exudates (Barraquio *et al.,* 2000). Several bacteria have the ability to attach to the root surfaces (rhizoplane) making them derive maximum benefit from root exudates. Many of them are more specialized, as they possess the ability to penetrate inside the root issues (endophytes) and have direct access to organic compounds present in the apoplast. By occupying this privileged endophytic location, bacteria do not have to face competition from their counterparts as encountered in the rhizosphere or in soil (Kanchana *et al.*, 2013).

Various strains of *Pseudomonasz fluorescens* has been identified as plant growth-promoting bacterial endophytes which colonize inside the roots and other plant parts. In a study conducted by Duijeff *et al.* (1997) an endophyte *Pseudomonas fluorescens* strain WCS417r colonized the root interior of tomato and induced resistance against fusarium wilt disease. The induction of disease resistance by strain WCS417r was hypothesized to be related to the extent of colonization of the internal root tissues.

Pseudomonas fluorescens FPT9601-T5, an endophytic plant growth-promoting rhizobacteria (PGPR) identified from tomato was inoculated in *Arabidopsis* and root colonization with *P. fluorescens* FPT9601-T5 promoted plant growth later than three weeks after inoculation and partially suppressed disease symptoms caused by *Pseudomonas syringae* (Wang *et al.,* 2005)

Pseudomonas fluorescens strain L321, an endophyte isolated from the bioenergy crop *Miscanthus giganteus* has shown to have excellent plant colonization abilities and it increased plant fresh weights and dry weights when trials were conducted in pots under greenhouse conditions using *Pisum sativum* L. The strain was found to be producing high levels of gluconic acid and phosphate solubilization (Oteino *et al.*, 2015).

2.2. ENDOPHYTES-A NOVEL TOOL FOR PLANT GROWTH PROMOTION

Endophytes are conventionally defined as bacteria or fungi that reside internally in plant tissues, can be isolated from the plant after surface disinfection, and cause no negative effects on plant growth (i.e., they are either beneficial or commensal) (Hallmann *et al.,* 1997). Recent molecular advances require that this definition be adjusted since an abundance of unculturable endophytes have been sequenced, but not isolated (Pereira *et al.*, 2011). The success of an introduced endophyte strain depends on its ability to survive and compete within the endophyte community colonizing the

plant (Conn and Franco, 2004). Extensive research has been done on the potential of root endophytes as inoculants for plant growth promotion.

Plant-growth-promoting bacterial endophytes (PGPBEs) facilitate plant growth via three inter-related mechanisms: phytostimulation, biofertilization, and biocontrol. Phytostimulation is the direct promotion of plant growth through the production of phytohormones (Bloemberg and Lugtenberg, 2001). Several endophytes that release ACC deaminase have been shown to increase plant growth, including *Arthrobacter* spp. and *Bacillus* spp. in chilli plants (Sziderics *et al.,* 2007). ACC deaminase production reduces abiotic stress by balancing plant ethylene-level production.

Fungal endophytes grow within plants without causing any disease symptoms. They are symptomless microorganisms living inside host plant that enhance host plant growth, improve nutrients uptake, reduce disease severity and enhance host tolerance to environmental stresses.

Fungal endophytes produce various biologically active metabolites and enzymes (Yuan *et al*., 2010). Among metabolites, plant hormones like GAs and auxin production is a new phenomenon in the endophytic fungi. Both GAs and auxin have been reported to play a pivotal role in plant growth (Waqas *et al.,* 2012). Waqas *et al.* (2012) isolated and examined two endophytic fungi for their potential to secrete phytohormones viz. gibberellins (GAs) and indoleacetic acid (IAA). The endophytic fungi isolated *Phoma glomerata* LWL2 and *Penicillium* sp. LWL3 significantly promoted the shoot and allied growth attributes rice.

Endophytic fungal association has been perceived beneficial to host-plants even during stress conditions. The endophytic fungi isolated *Phoma glomerata* LWL2 and *Penicillium* sp. LWL3 application and endophytic-association with host-cucumber plants significantly increased the plant biomass and related growth parameters under sodium chloride and polyethylene glycol induced salinity and drought stress. The

endophyte-infection significantly modulated stress through down-regulated abscisic acid, altered jasmonic acid, and elevated salicylic acid contents as compared to control (Waqas *et al.*, 2012)

The potential role of the endophyte and its biologically active metabolites in its association with its host has been investigated. Production of vincristine, an anticancer drug obtained from the plant *Catharanthus roseus* (Apocynaceae), was enhanced by *Fusarium oxysporum* endophytic in the same plant (Lingqi *et al.* 2000).

Neotyphodium uncinatum, a common endophyte of the grass *Festuca pratensis*, was found to have the full biosynthetic capacity for some of the most common loline alkaloids, originally obtained from the plant (Blankenship *et a*l. 2001).

A higher proportion of the endophytic fungi exhibited biological activity than the soil isolates did and the biological activity of the fungi is attributed to the production of variety of secondary metabolites like terpenoids, steroids, xanthones, chinones, phenols, isocumarines, benzopyranones, tetralones, cytochalasines, and enniatines (Schulz *et al.*, 2002).

It has been a known factor that these endophytic fungi, residing inside host confer abiotic stress tolerance. Rodriguez *et al.*, (2008) conducted a study in *Dichanthelium lanuginosum,* a grass species that thrives in the hot geothermal soils with root zone temperatures of 57°C were found to be colonized with fungal endophyte, *Curvularia protuberate* and it was also observed that uncolonized plants could not tolerate root zone temperatures above 38◦C, but colonized plants tolerated temperatures up to 65◦C over a 10-day period.

2.2.1. *Piriformospora indica* - A PLANT GROWTH PROMOTING ROOT ENDOPHYTIC FUNGI

Piriformospora indica is a well-established fungal endophyte, which confers benefits such as plant growth promotion and increases the resistance against pathogens in a broad range of host plants. It belongs to the newly formed family Sebacinaceae in the order of Sebacinales with a close resemblance with the Arbuscular Mycorrhizal fungi (Weiss *et al.,* 2004). It is a novel phyto-promotional, biotropic, mutualistic root endophytic fungus, found to colonize in roots of many commercially important crop plants which was initially isolated from the rhizosphere of the xerophytic plants in the Thar Desert, India (Verma *et al*., 1998).

The cultivation of *P. indica* could be performed axenically in conventional media such as PDA / PDB. *P. indica* is very simple to grow in a bioreactor and can be formulated effectively (Singh *et al.,*2003;Qiang *et al.,* 2011). Root colonization by *P. indica* results in an increase in plant growth, early flowering, higher seed yield, alteration in the secondary metabolites, and adaptation to abiotic and biotic stresses (Varma *et al.,* 2012)

Varma *et al.* (1999) observed that *P. indica* tremendously improved the growth and overall biomass production of different plants such as herbaceous monocots and dicots, including medicinal plants like *Bacopa monnieri, Artemisia annua, Withamnia somnifera* and many other economically important crops. The endophytic fungus has been reported to increase the production of spilanthol, an endogenous content of medicinal plants besides plant growth promotion (Rai *et al*., 2004). *P. indica* supports the uptake of nitrogen by plants through the enhanced expression of nitrate reductase in plant roots colonized by the fungus (Sherameti *et al*., 2005).

P. indica exhibited a potential effect in inhibiting the ethylene signaling process which helps in reducing the ethylene content in plants thereby contributing to plant

growth promotion (Barazani *et al.*, 2007). Photosynthetic efficiency of maize plants was triggered by the application of *P. indica* which was observed when chlorophyll fluorescence was analyzed (Rai *et al.*, 2008).

A steep increase in the vegetative growth by 50 per cent was noticed in tomato seedlings raised by inoculating the fungus by root dipping (Fakhro *et al.*, 2010).

Colonization of *P. indica* in black pepper root system and the improved growth in tissue cultured plantlets on inoculation with *P. indica* has been reported by Anith *et al.* (2011).

P. indica is considered as one of the powerful tools for crop improvement since it helps in mediating the improvement in crop productivity under unfavorable environmental conditions (Ansari *et al.*, 2014).

It acts as a potential biological tool for hardening the tissue culture-raised plants and triggers the production of secondary metabolites, plant biomass, early flowering, growth promotion and seed production (Verma *et al.*, 1998; Yadav *et al.*, 2010; Das *et al.*, 2012; Anith *et al.*, 2018).

Plants colonized by *P. indica* tend to show an increased concentration of proline which explains the osmotic stress tolerance by the plants (Zarea *et al.*, 2012). *Bacopa monieri* when cultivated with the addition of *P. indica*, augmented the growth along with an elevated level of bacoside production and increased antioxidant activity (Prasad *et al.*, 2013). An increase of 28.8 per cent in aristolochic acid content was reported in *Aristolochia elegans* treated with culture filtrate of *P. indica* (Bagde *et al.*, 2013).

Triticum aestivum exhibited positive correlation with abiotic stress when colonized by *P. indica*. There was an increase in plant growth when the salt concentration rose beyond the normal level (Zarea *et al.*, 2012).

Justice *et al.* (2018) reported potential enhancement of adventitious root formation as well as increase in root weight in the flowering plants like crossandra, dahlia and poinsettia when the cuttings were planted in rooting medium amended with *P. indica*.

Earliness in flowering, yield enhancement and improved piperine content in the berries of black pepper plants has been reported by Anith *et al.* (2018). Inoculation of the fungus to miniature plants referred to as "bush pepper" in pot culture conditions improved both fresh and dry berry yield many fold.

Besides growth promotion, *P. indica* was found to have efficient disease control over virulent root and seed pathogens. Antioxidant system in plants are triggered by the endophytic fungus and helped the inoculated plants to tide over biotic and abiotic stress (Waller *et al.,* 2005; Deshmukh and Kogel, 2007; Druege *et al.*, 2007). *P. indica* was able to suppress the incidence of root knot nematode infestation in tomato effectively (Varkey *et al.* 2018).

2.2.2. COMBINED INOCULATION OF *Piriformospora indica* WITH OTHER ORGANISMS

For increasing the spectrum of action and efficiency of bio-inoculants, they can be used as mixed inoculum or consortium with more than one bio-agents as a formulation (Pierson and Weller, 1994; Vidyasekarn and Muthamilan, 1995; Schisler *et al.*, 1997; Janisiewicz, 1988; Slininger *et al.*, 2010).

Meena *et al.* (2010) reported that the combination of *P. indica* and phosphate solubilizing bacteria *Pseudomonas striata* when inoculated on to chick pea showed an improvement in the growth of the plant.

P. indica, the fungal endophyte has been used as a mixed inoculum with PGPR strains for improving growth response and disease suppression in crops like mung bean

and tomato. The application of a talc based consortium of two strains of *Pseudomonas* with *P. indica* when applied to tomato plants in controlled glass house experiment showed an increment of 8.8 fold in dry root weight and 8.6 in dry shoot weight. A similar observation was noticed in the field also (Sarma *et al.*, 2011).

A consortium containing *P. indica* and two Pseudomonad strains (R62 and R81) when applied to mung bean resulted in increased growth under glass house and field conditions. Increase in dry root and shoot weight, number of nodules produced and number of pods harvested were noted in the consortial treatment compared to the control (Kumar *et al.*, 2012).

Synergistic effects of co-inoculation of phosphate solubilizing bacteria and the endophytic fungus has been demonstrated in pot culture experiments involving chick pea (Saxena *et al.*, 2015).

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

Significant improvement in the growth, symbiotic parameters and grain yield were observed in chick pea inoculated with a consortium of *Mesorhizobium cier*, *P. indica* and *Pseudomonas argentinensis*. Presence of high level of leghaemoglobin and chlorophyll content also was observed in the plants inoculated with the consortium compared to the uninoculated control (Mansotra *et al.*, 2015).

Arora *et al.* (2018) reported that dual biological consortium containing *P. indica* and *Azotobacter chroococcum* enhanced artemisin content in the medical plant *Artemisia annua*. The overall productivity was increased due to increased contents of chlorophyll and nutrients such as nitrogen and phosphorous. Further it was reported that under *in vitro* conditions also enhancement of metabolite content in *Artemisia*

annua occurred on dual inoculation with the bacterial and fungal bioagents (Arora *et al.*, 2018).

2.3 COCULTURING OF BENEFICIAL MICROORGANISMS

Growing two or more organism in a single fermentation system is termed as co-culturing. Various studies have reported that, co-culturing resulted in various advantages like production of metabolites that can be used as drugs. It was also reported that co-culture can increases the plant growth promoting ability and biocontrol efficiency of PGPR.

Rice *et al.* (1994) observed an increased population of bacteria in a co-culture than monoculture of *Rhizobium meliloti* and phosphate solubilizing bacteria *Pencillium* sp.in sterile peat.

Nitrogen-fixation by *Azospirillum brasilense* CD is promoted when co-cultured with a mangrove rhizosphere bacterium *(Staphylococcus sp.)* (Holguin and Bhashan, 1996).

Co-culturing sometimes results in change in morphology of organisms, especially fungi. In a co-culture with *Bacillus subtilis* and *Fonsecaea pedrosoi*, the interaction among the microorganisms cause morphological modification in the fungus (Machado *et al.*, 2010).

Co-culturing of PGPR will enhance its growth promotion and bioconrolling efficiency by increasing its colonizing ability in plants, producing various metabolites and biofilm formation. Jayasinghearchchi and Seneviratne in 2010 reported that a mushroom fungi *Pleurotus ostreatus* co-cultured with *Pseudomonas fluorescens* improved the endophyte colonization of tomato through biofilm formation.

Mixture of various strains of *Pseudomonas fluorescens* and *Enterobacteria cloacae* were produced by co-culturing it in a vessel and applied to stored potato and observed a decrease in disease percentage in potatos in storage (Slininger *et al.,* 2010).

When co-cultured, bacterial-fungal interactions give rise to enhanced effects of biosynthesis compared to their monocultures. Teles *et al.,* (2012) reported that co-culturing with *Salmonella tyohiurium* modulated the antimicrobial metabolite produced by *Paecilomyces lilacinus.*

In study of co-culturing of PGPR, *Bacillus pumilis* with fungal root endophyte *P. indica,* in coconut water resulted in enhanced growth of tomato seedling compared to individual application of the organism and mixed inoculation of separately grown bacteria and fungi. It was also observed that the root colonization pattern of *P. indica* in plants treated with co-cultured inoculum was different from other treatments (Anith *et al.*, 2015)

Co-culturing requires a single medium that could support the growth of the component microbial strains. It should be rich with various source of sugars, amino acid,enzymes etc. for growing a variety of organisms like bacteria and fungi. A number of studies have suggested coconut water, naturally available and cheap product, as highly potential medium for growing microorganisms.

Anandaraj *et al.* (1997) reported mature coconut water as an excellent option for mass multiplication of various biocontrol agent as it is rich in nutrients.

Use of coconut water as a biofermentor or as an additive to the medium for growing microorganisms increases the biomass production and the level of desirable product obtained. In case of production of docosahexaenoic acid the level of acid obtained and the biomass of *Schizochtrium mangrovei* Sk-02 was found to be almost 50 % higher in coconut water supplemented medium compared to the non-supplemented culture (Unagul *et al.*, 2007). It was also reported that coconut water is

a suitable medium for production δ endotoxin by *Bacillus thuringiensis* var. *israelensis,* a mosquitocidal agent (Prabhakaran *et al.*, 2008).

PGPR grown in coconut water gives additional advantages in plant growth promotion compared to a PGPR grown in conventional medium. Population of PGPR like *Pseudomonas fluorescens* PN026R and *Bacillus pumilis* SE34 was higher in the developed roots of pepper cuttings treated with bacterial culture grown in coconut water, than that grown in conventional media (Anith, 2009).

When tomato seeds were treated with the same bacterial strains grown in coconut water, there was an enhanced growth promotion observed compared to bacteria grown in conventional media (Anith, 2009). Same trend was observed when the PGPR *Pseudomonas fluorescens* AMB8 grown in coconut water along with an additive, polyvinylpyrrolidone was used for seed treatment in chilli and tomato (Anith *et al.*, 2016).

Multiplication of PGPR in coconut water makes bio-fertilizer production more farmer friendly. PGPR like *Bacillus pumilis* and *Pseudomonas fluorescens* PN026R has been reported to be multiplied in coconut water and the growth pattern of the bacterial strains were comparable to conventional liquid media (Anith, 2009).

It was also reported that application of co-culture suspension of *Bacillus pumilis* and fungal root endophyte *Piriformospora indica* in coconut water has shown maximum value of growth promotion in tomato seedlings (Anith *et al.*, 2015).

Materials and Methods

3. MATERIALS AND METHOD

The experiments in the research programme entitled "Growth promotion in chilli (*Capsicum annuum* L.) on inoculation with *Pseudomonas fluorescens* and *Piriformospora indica*" were carried out in the Department of Agricultural Microbiology, College of Agriculture, Vellayani during 2017-2019. The details of materials used and methods employed in the study are described below.

3.1. MICROBIAL BIOAGENTS

3.1.1. Rhizobacteria

Pseudomonas fluorescens PN026 and *Pseudomonas fluorescens* AMB8 were obtained from the Department of Agricultural Microbiology, College of Agriculture, Vellayni. The revived isolates of Pseudomonas strains from the glycerol cultures were routinely cultivated on King's medium B agar / King's medium B broth at 28°C. The purified isolates were preserved on KB slants for further use.

3.1.2. Fungal Endophytes

Piriformospoara indica kindly provided by Dr. Ajith Varma, former Professor, Jawaharlal Nehru University, New Delhi and available at the Department of Agricultural Microbiology, College of Agriculture, Vellayani was transferred to PDA plates and incubated at 28°C. Further it as purified by the hyphal tip method of PDA plates and preserved on PDA slants under refrigeration (Plate 1).

3.2. PLANT VARIETY

The plant variety used was Vellayani Athulya (KAU, Department of Vegetable Science, College of Agriculture, Vellayani) (Plate 2).

Plate 1. Fungal root endophyte,
Piriformospora indica

Plate 2. Chilli variety Vellayani
Athulya

3.3. *In vitro* ANTAGONISM BETWEEN *Piriformospora indica* AND BACTERIAL STRAIN

3.3.1. Direct Antagonism

Compatibility of *Piriformospoara indica* with *Pseudomonas fluorescens* strains was evaluated by dual culture plate assay in PDA and coconut water agar (CWA).

3.2.1.1. Dual culture plate assay

For preparing CWA, fresh coconut water was procured and filtered through muslin cloth to get rid of the suspended particles and debris. 300 ml of coconut water were transferred to a 500 ml Erlenmeyer flask, the pH was adjusted to 6.5 and 2 % agar was added and sterilized by autoclaving at 121 °C for 20 min.

Single colonies of *Pseudomonas* strains were obtained by streak plating on King's medium B agar. Mycelial disc (8 mm dia) was cut from the 10 day old culture of *P.indica* grown on PDA plates and transferred to the centre of fresh PDA plate and CWA plate. When the fungal growth reached a diameter of 5 cm, each of the test organism each of the test organism was streaked as a band (5cm) separately on two sides of the PDA plates and CWA plates at a distance of 2 cm away from the periphery. Control plates were also maintained with *P.indica* alone. Plates were incubated at 28°C for seven days. Observations were recorded by measuring the inhibition zone if any (Anith *et al.*, 2015).

3.3.2. Indirect Antagonism

Antagonistic effect of the culture filtrate of the bacterial bioagents against the endophytic fungus was assessed by agar well diffusion method and disc diffusion method.

3.3.2.1. Extraction of culture filtrate

From the pure culture of the bacterial bioagents, a loopful of bacterial cells was transferred to King's medium B broth. It was incubated overnight in incubator shaker (110 rpm) at 28°C. Ten ml of the broth culture from each of the strain was centrifuged at 10,000 rpm for five minutes in sterile polypropylene tube. The supernatant was aseptically collected and filter sterilized using a 0.2 µ nitrocellulose bacteriological filter. The filtrate was aseptically collected and stored at 4°C for further use.

3.3.2.2. Agar Well Diffusion Method

Mycelial disc (8 mm dia) was cut from the 10 day old culture of *P. indica* grown on PDA plates and transferred to the centre of fresh PDA plate. Wells (8 mm dia) were cut at two opposite edges of the plate using a sterile cork borer. The wells were partially filled with 100 *µl* of molten agar. Once the well was sealed properly, 100 *µl each of the* culture filtrate of each test organism was added to the wells *and incubated for* 48 h at 28°C. Three replications were maintained for each of the organisms. *The inhibition zone from the well was measured (*Balouiri *et al.*, 2016*).*

3.3.2.3. Disc Diffusion Method

Mycelial disc (8 mm dia) was cut from the 10 day old culture of *P. indica* grown on PDA plates and transferred to the centre of fresh PDA plate. *Sterile filter paper discs (5 mm dia) were soaked with* ten *µl of the culture filtrate. The discs were dried in a laminar air flow chamber and placed at two opposite edges of the Petri plate containing* the fungus. *Plates were then incubated for a* period of 48 h at 28°C. Three replications were maintained for each of the organism. Inhibition zone from the filter paper disc was measured (Nawangsih *et al.,* 2011).

Plate 3. Co-culturing of *P. indica* and *P. fluorescens* strain in a single fermentation system

3.4. CO-CULTURE EXPERIMENT

For preparing autoclaved coconut water (ACW), fresh coconut water was procured and filtered through muslin cloth to get rid of the suspended particles and debris. 100 ml of coconut water were transferred to a 250 ml Erlenmeyer flask, the pH was adjusted to 6.5 and the solution was sterilized by autoclaving at 121 °C for 20 min. PDB (pH of 6.5), 100 ml was sterilized in 250 ml Erlenmeyer flask and both the media were inoculated with two mycelial plugs (8 mm dia) of *Piriformospora indica* obtained from PDA plates previously grown for 10 days. Bacterial strains were streaked out for single colonies on King's medium B agar plate. Cells from a single colony were pooled in one ml of sterile distilled water and 200 µl of the bacterial suspension was aseptically added to flasks of PDB and ACW wherein *P. indica* had been growing since 10 days. The initial population of the bacteria added to the flasks was determined by dilution plating on King's B agar medium immediately after inoculation. The flasks were further incubated under agitation (150 rpm) for 48 h and the population of the bacteria was determined at 24 h intervals by dilution plating on King's medium B agar. The bacterial population from five flasks was independently assessed for both growth media. Growth of the bacteria in fresh PDB, ACW and King's B broth was taken as baseline to determine the efficiency of the co-culture in supporting bacterial growth (Anith *et al.*, 2015) (Plate 3).

3.5. *IN VIVO* STUDIES:

3.5.1. Plant growth promotion in chilli (pot culture experiment)

Vermiculite was used as planting medium in the pro-trays. It was sterilized by autoclaving at 121^0C for 1 h each for three consecutive days. Pro-trays (50 cells; each cell having a dia of 5 cm) were filled with the sterile potting mixture. Seeds of chilli were surface sterilized in one percent sodium hypochlorite aqueous solution for 3 minutes in a laminar air flow chamber. The seeds were further washed thrice with sterile distilled water.

Pseudomonas strains were heavily cross streaked on King's medium B agar. After 24 h of incubation, the plates were drenched with 10 ml sterile distilled water and the bacterial growth was suspended in it by using a sterile glass spreader. The suspension was collected aseptically in sterile glass vials. The OD of the suspension was adjusted to 0.6 at 660 nm using sterile distilled water so that the suspension contains approximately 10^8cfu ml^{-1}. Bacterization was done by soaking the surface sterilized seeds in bacterial cultures for 20 minutes prior to seeding.

P. indica mycelium was incorporated into the planting medium before filling the pro-tray cavities. For this, mycelium of the fungal endophyte grown (as mentioned in the section 3.3.2.1.) for 15 days in a 250 ml flask containing 100 ml PDB medium was collected by filtering the contents of the flask through a muslin cloth. The same was weighed and mixed thoroughly with sterile planting medium @ one percent (w/v).

Two seeds were planted per cavity of pro-tray and further thinned to single seedling after germination. Plants were grown in a net house with natural ventilation, sunlight as light source with 50 percent shade. Seedlings were irrigated with tap water twice daily. Once in 10 days, fertigation was provided by pouring 10 ml of one per cent water soluble fertilizer solution (N:P:K - 17:17:17) per cavity starting from first week after seeding. Plants were kept for 25 days in the nursery (Plate 4). Twenty five day-old seedlings were transplanted to pots (15 cm dia) filled with one kg each of potting mixture (soil, sand and cow dung in the ratio 2:1:1) (Plate 5 and 6).

T1: *Pseudomonas fluorescens* PN026

T2: *Pseudomonas fluorescens* AMB8

T3: *Piriformospora indica*

T4: Combination of *Pseudomonas fluorescens* PN026 and *Piriformospora indica*

Plate 4: Nursery production of chilli plants in pro-tray cavities

Plate 5. General view of the experimental plot at the time of transplanting

Plate 6. General view of the experimental plot at the time of harvesting

T5: combination of *Pseudomonas fluorescens* AMB8 and *Piriformospora indica*

T6: Uninoculated control

T7: Co-cultured *P. indica* and *Pseudomonas fluorescens* PN026

T8: Co-cultured *P. indica* and *Pseudomonas fluorescens* AMB8

Design: CRD

Replications: 3

Number of plants per replication: 5

3.5.2. Biometric Observation

Biometric observations were taken at an interval of fifteen days. The observations recorded were; plant height (cm), number of leaves, number of branches, number of fruits/plant, fresh fruit yield (g/plant), days to flowering, days to fruit set, pest and disease occurrence, if any and *P. indica* root colonization (%). After 80 days, destructive sampling was done to assess fresh shoot weight (g), dry shoot weight (g), fresh root weight (g) and dry root weight (g). The dry weights were taken after drying plant samples for three days at 60°C in a hot air oven

3.6. ROOT COLONIZATION BY *Piriformospora indica*

The plants treated with *Piriformospora indica* were assessed for root colonization by the endophytic fungus. 30 days after plant growth, five plants from each treatment were uprooted without damaging the roots (Anith *et al.*, 2015). The root system was washed in running tap water to get rid of the adhering planting medium. They were then cut into small bits of one cm length. The bits were softened by boiling in 10 per cent potassium hydroxide (KOH) for five minutes. KOH was removed by washing with distilled water. Roots were then acidified with 1N HCl for five minutes

and directly transferred to the staining solution, lactophenol-tryphan blue for 10 minutes. Destaining with lactophenol solution for 10 minutes was done prior to examination under a compound bright field microscope. Presence of chlamydospores was taken as a positive indication of root colonization. The percentage root colonization was calculated using the formula;

$$\text{Percentage root colonization} = \frac{\text{No. of root bits with chlamydospores}}{\text{Total number of root bits observed}} \times 100$$

3.7. STATISTICAL ANALYSIS

Statistical analysis was done using the package available with the online portal of IASRI, New Delhi. The means were compared using Least Significant Difference (LSD) at 5 per cent level of significance using ANOVA.

Results

4. RESULTS

The data obtained under the present investigation on "Growth promotion in chilli (*Capsicum annuum* L.) on inoculation with *Pseudomonas fluorescens* and *Piriformospora indica*" were analyzed and the results obtained are presented in this chapter under following headings.

4.1. *In vitro* ANTAGONISM BETWEEN *Piriformospora indica* AND BACERIAL STRAIN

4.1.1. Direct Antagonism by Dual Culture Plate Assay

In vitro interaction of bacterial bioagents and *Piriformospora indica* using dual culture plate assay was done to assess the compatibility between them (Plate 7). The zone of inhibition was measured in 5th and 7th day after the bacterial inoculation. In PDA plates both *Pseudomonas fluorescens* PN026 and *Pseudomonas fluorescens* AMB8 inhibited the growth of *Piriformospora indica* with zone of inhibition of 1.16 mm and 1.50 mm respectively in 7th day after bacterial inoculation. In CWA plates *P. fluorescens* PN026 had no inhibition on *P. indica* and *P. fluorescens* AMB8 had a reduced inhibition of 0.67 mm compared to PDA plates (Table 1, Plate 7 and 8)

4.1.2. Indirect Antagonism by Culture Filtrate of Bacterial Strains against *P. indica*

4.1.2.1. Agar Well Diffusion Method

The culture filtrates of both bacterial strains exhibited antagonism against the fungus *Piriformospora indica.* On 7th day after bacterial inoculation zone of inhibition was the higher in *P. fluorescens* AMB 8 (0.84 mm) than that of *P. fluorescens* PN026 (0.17 mm) (Table2, Plate9)

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

Bacterial strain	ZONE OF INHIBITION (mm) *			
	PDA*		CWA*	
	5TH DAY	7TH DAY	5TH DAY	7TH DAY
Pseudomonas fluorescens PN026	4.34	1.16	0.67	nil
Pseudomons fluorescens AMB8	5.67	1.50	2.34	0.67

*PDA = Potato dextrose agar
*CWA= Coconut water agar

*Mean of eight observation from 4 dual culture plates. Each plate represents single replication (n=4)

4.1.2.2. Disc Diffusion Method

The discs impregnated with culture filtrate of *P. fluorescens* strains exhibited antagonistic activity against *Piriformospora indica* by producing inhibition zone (Plate 9). Zone of inhibition was maximum for *P. fluorescens* AMB 8 (3 mm) and *P. fluorescens* PN026 showed zone of inhibition of 0.83 mm on 7th day after bacterial inoculation (Table 3 and Plate 9).,

4.2. CO-CULTURE EXPERIMENT

Co-culturing of the bacterial bioagents with the endophytic fungus *Piriformospora indica* showed varying levels of population buildup of the bacteria after 24 h and 48 h of incubation (Table 4 and 5).When 10 day-old cultures of the fungus in ACW and PDB were inoculated with the bacterial strains, the former medium supported the growth of the bacteria similarly to fungus free culture and King's medium B broth. Both monoculture and co-culture in ACW resulted in achieving a population of 10^{10} cfu/ml from an initial inoculum of 10^5 cfu/ml. On the other hand co-cultivation PDB led to a decline in bacterial population. In ACW *Pseudomonas fluorescens* PN026 grew similar to that of the conventional medium King's medium B broth and when co-cultured with fungi the population build up was comparable with conventional medium and higher than that of the monoculture of the bacteria. In case of *Pseudomonas fluorescens* AMB8 same trend was observed except that the population in co-culturing was lower than that of the monoculture of the bacteria.

Table 2. Antagonistic assay by agar well diffusion method

Bacterial strain	ZONE OF INHIBITION (mm) *		
	5^{TH} DAY	6^{TH} DAY	7^{TH} DAY
Pseudomonas fluorescens PN026	2.17	1.34	0.17
Pseudomonas fluorescens AMB8	3.00	1.84	0.84

Table 3. Antagonistic assay by filter paper disc diffusion method

Bacterial strain	ZONE OF INHIBITION (mm) *		
	5^{TH} DAY	6^{TH} DAY	7^{TH} DAY
Pseudomonas fluorescens PN026	3.88	1.83	0.83
Pseudomonas fluorescens AMB8	4.50	3.50	3.00

*Mean of eight observation from 3 plates. Each plate represents single replication (n=3)

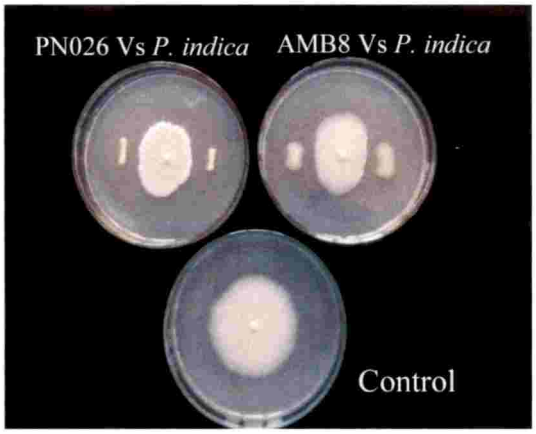


Plate 7. *In vitro* assessment of compatibility between bacterial bioagents and *P. indica* in PDA plates

Plate 8. *In vitro* assessment of compatibility between bacterial bioagents and *P. indica* in CWA plates

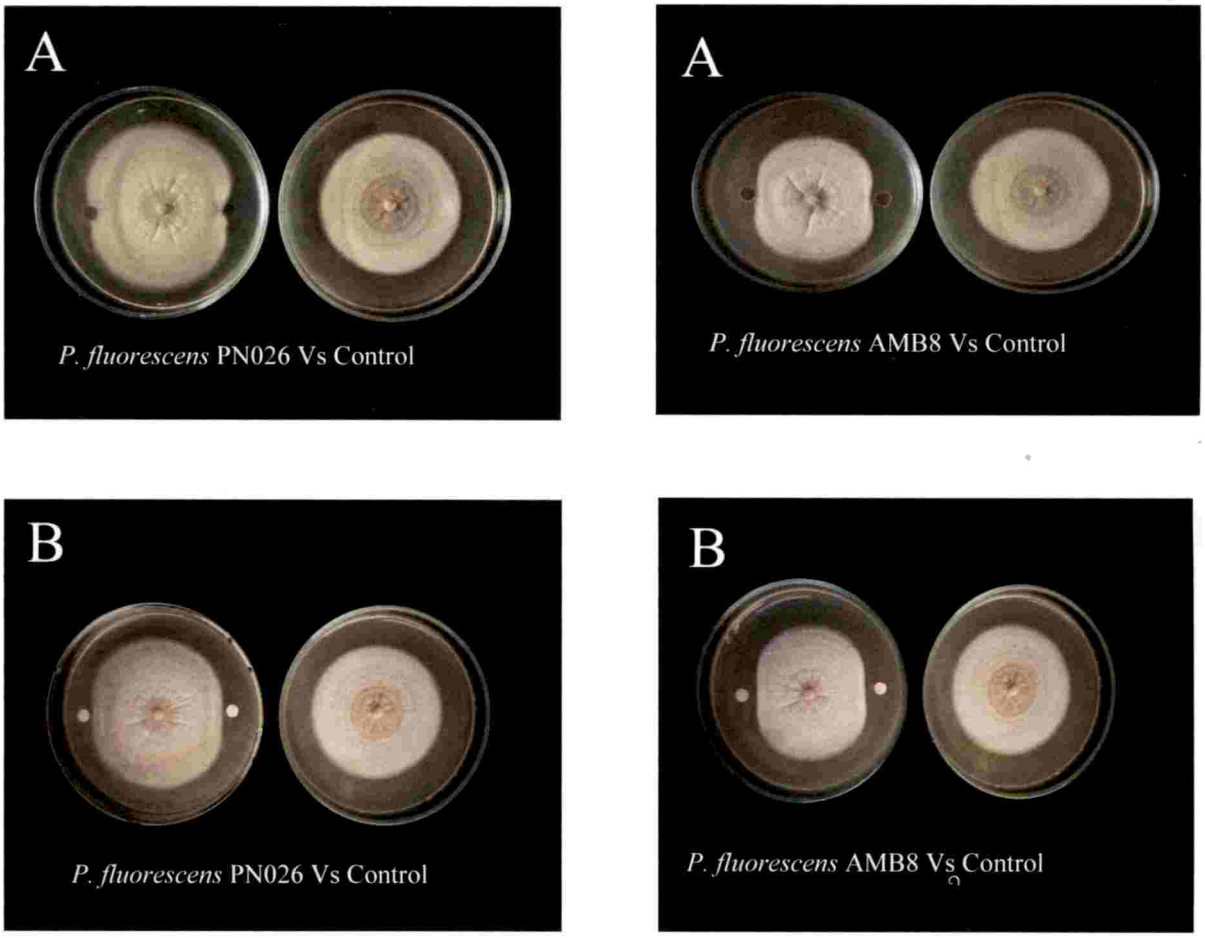


Plate 9. Indirect methods used for checking antagonism of bacterial bioagents against the fumgal endophyte *Piriformospora indica* A. Agar well diffusion method; B. Disc diffusion method

Table 4. Population buildup of *Pseudomonas fluorescens* PN026 (cfu ml^{-1}) in different media and cultural condition

Type of inoculation	Population buildup of *Pseudomonas fluorescens* PN026 (cfu ml^{-1})		
	Time of population assessment		
	0 h	24 h	48 h
***Pseudomonas fluorescens* PN026 alone**			
PDB	1.17 x 10^5	1.00 x 10^7	1.50 x10^6
ACW	1.84 x 10^5	5.57 x 10^7	1.45 x 10^{10}
KB	2.74 x 10^5	6.10 x 10^7	1.65 x 10^{10}
***P. indica* and *Pseudomonas fluorescens* PN026 coculture**			
PDB	3.30 x 10^4	8.60 x 10^4	6.60 x 10^4
ACW	1.18 x 10^5	6.1 x 10^7	1.65 x 10^{10}

*Mean population from five flasks. Each flask represents single replication (n = 5)
PDB = Potato dextrose broth
ACW = Autoclaved coconut water
KB = King's B media

Table 5. Population buildup of *Pseudomonas fluorescens* AMB8 (cfu ml^{-1}) in different media and cultural condition

Type of inoculation	Population buildup of *Pseudomonas fluorescens* AMB8 (cfu ml^{-1})		
	Time of population assessment		
	0 h	24 h	48 h
***Pseudomonas fluorescens* AMB8 alone**			
PDB	1.35×10^5	1.60×10^6	4.40×10^6
ACW	4.40×10^5	5.57×10^7	1.45×10^{10}
KB	7.56×10^5	3.67×10^9	2.77×10^{10}
***P. indica* and *Pseudomonas fluorescens* AMB8 coculture**			
PDB	5.00×10^4	4.97×10^4	5.30×10^4
ACW	1.74×10^5	1.77×10^7	1.05×10^{10}

*Mean population from five flasks. Each flask represents single replication (n = 5)
PDB = Potato dextrose broth
ACW = Autoclaved coconut water
KB = King's B media

Table 6. Biometric observation on chilli treated with various bioagents on variety Vellayani Athulya - Plant height (cm)

Treatments	Plant height (cm)				
	Time of observation				
	15 DAT	30 DAT	45 DAT	60 DAT	75 DAT
***Pseudomonas fluorescens* PN026**	13.93	22.1	27.04	34.93	40.34
***Pseudomonas fluorescens* AMB8**	11.68	22.17	26.96	33.04	38.50
Piriformospora indica	13.45	23.58	25.42	32.00	38.51
Pseudomonas fluorescens* PN026 and *Piriformospora indica	16.80	24.46	27.34	34.63	41.88
Pseudomonas fluorescens* AMB8 and *Piriformospora indica	13.92	24.92	27.34	33.42	39.34
Co-cultured *Pseudomonas fluorescens* PN026 and *Piriformospora indica*	13.14	23.00	27.25	35.67	42.17
Co-cultured *Pseudomonas fluorescens* AMB8 and *Piriformospora indica*	13.17	21.17	22.87	28.92	36.08
Uninoculated control	14.29	20.25	24.71	31.73	38.34
SEm (±)	0.97	1.87	0.11	2.04	2.03
CD (0.05)	NS	NS	NS	NS	NS

*Mean of three replication having five plants each.

Table 7. Biometric observation on chilli treated with various bioagents on variety Vellayani– No. of leaves

Treatments	No. of leaves				
	Time of observation				
	15 DAT	30 DAT	45 DAT	60 DAT	75 DAT
***Pseudomonas fluorescens* PN026**	11.50	21.67	34.00	45.38	54.17
***Pseudomonas fluorescens* AMB8**	10.50	18.67	37.08	43.58	51.34
Piriformospora indica	12.83	21.84	41.83	62.37	65.54
Pseudomonas fluorescens* PN026 and *Piriformospora indica	14.04	26.01	46.79	62.71	68.34
Pseudomonas fluorescens* AMB8 and *Piriformospora indica	12.17	19.24	34.25	39.83	48.08
Co-cultured *Pseudomonas fluorescens* PN026 and *Piriformospora indica*	11.17	26.25	45.04	64.09	65.50
Co-cultured *Pseudomonas fluorescens* AMB8 and *Piriformospora indica*	13.42	21.25	27.79	37.63	48.50
Uninoculated control	13.25	17.50	33.83	38.00	45.92
SEm (±)	1.12	2.47	2.56	3.85	3.79
CD (0.05)	NS	NS	7.50	11.31	11.12

*Mean of three replication having five plants each

Table 8. Biometric observation on chilli treated with various bioagents on variety Vellayani Athulya –No .of branches

Treatments	No .of branches				
	Time of observation				
	15 DAT	30 DAT	45 DAT	60 DAT	75 DAT
***Pseudomonas fluorescens* PN026**	1.00	2.50	3.71	5.67	12.17
***Pseudomonas fluorescens* AMB8**	1.04	1.92	3.92	6.17	11.58
Piriformospora indica	1.04	2.34	4.25	6.58	10.74
Pseudomonas fluorescens* PN026 and *Piriformospora indica	1.00	3.75	4.04	7.17	15.75
Pseudomonas fluorescens* AMB8 and *Piriformospora indica	1.00	1.75	2.75	3.00	9.92
Co-cultured *Pseudomonas fluorescens* PN026 and *Piriformospora indica*	1.04	2.83	3.42	6.58	14.67
Co-cultured *Pseudomonas fluorescens* AMB8 and *Piriformospora indica*	1.12	1.92	2.42	4.67	10.08
Uninoculated control	1.12	2.17	3.33	4.42	7.25
SEm (±)	0.03	0.55	0.48	0.83	1.32
CD (0.05)	0.09	NS	NS	2.44	3.89

*Mean of three replication having five plants each

Table 9. Biometric observation on chilli treated with various bioagents on variety Vellayani Athulya after harvest (80 DAT) – Days to first flowering

Treatments	Days to first flowering
Pseudomonas fluorescens PN026	35.67
Pseudomonas fluorescens AMB8	38.25
Piriformospora indica	35.34
Pseudomonas fluorescens PN026 and *Piriformospora indica*	34.88
Pseudomonas fluorescens AMB8 and *Piriformospora indica*	36.50
Co-cultured *Pseudomonas fluorescens* PN026 and *Piriformospora indica*	31.75
Co-cultured *Pseudomonas fluorescens* AMB8 and *Piriformospora indica*	35.33
Uninoculated control	21.61
SEm (±)	1.20
CD (0.05)	3.52

*Mean of three replication having five plants each

Table 10. Biometric observation on chilli treated with various bioagents on variety Vellayani Athulya after harvest (80 DAT) – Days to fruit set

Treatments	Days to fruit set
Pseudomonas fluorescens PN026	70.58
Pseudomonas fluorescens AMB8	68.00
Piriformospora indica	61.59
Pseudomonas fluorescens PN026 and *Piriformospora indica*	63.75
Pseudomonas fluorescens AMB8 and *Piriformospora indica*	66.17
Co-cultured *Pseudomonas fluorescens* PN026 and *Piriformospora indica*	62.25
Co-cultured *Pseudomonas fluorescens* AMB8 and *Piriformospora indica*	66.34
Uninoculated control	62.84
SEm (±)	2.20
CD (0.05)	NS

*Mean of three replication having five plants each

Table 11. Biometric observation on chilli treated with various bioagents on variety Vellayani Athulya after harvest (80 DAT) – No. of fruits per plant

Treatments	No. of fruits /plant
Pseudomonas fluorescens PN026	8.71
Pseudomonas fluorescens AMB8	8.13
Piriformospora indica	9.76
Pseudomonas fluorescens PN026 and *Piriformospora indica*	10.25
Pseudomonas fluorescens AMB8 and *Piriformospora indica*	8.42
Co-cultured *Pseudomonas fluorescens* PN026 and *Piriformospora indica*	11.25
Co-cultured *Pseudomonas fluorescens* AMB8 and *Piriformospora indica*	7.29
Uninoculated control	8.09
SEm (±)	0.40
CD (0.05)	1.17

*Mean of three replication having five plants each

4.3. *IN VIVO* STUDIES:

4.3.1. PLANT GROWTH PROMOTION BY RHIZOBACTERIA AND FUNGAL ENDOPHYTE IN VARIETY VELLAYANI ATHULYA (POT CULTURE EXPERIMENT)

Growth parameters like plant height, number of leaves and number of branches were measured in fifteen days interval after transplanting. With respect to plant height there was no significant difference among the treatments. Maximum values were obtained in the treatment of mixed inoculation of *P. indica* and *P. fluorescens* PN026. 60 days after transplanting onwards maximum value was recorded in the treatment of co- cultured *P. indica* and *P. fluorescens* PN026 (Table 6).

Highest number of leaves were recorded in the plants treated either with mixed or co-cultured combination of *P. indica* and *P. fluorescens* PN026. In the observation taken in 45^{th} day and 75^{th} day after transplanting higher number of leaves (46.79 and 68.45 respectively) were recorded in the treatment with mixed inoculation of *P. indica* and *P. fluorescens* PN026 and the values were statistically on par with those treated with co-cultured *P. indica* and *P. fluorescens* PN026 and *P.indica* alone. On 60^{th} day after transplanting, highest number of leaves (64.08) were observed in the treatment with co-cultured *P. indica* and *P. fluorescens* PN026 (Table 7).

A significantly increasing trend was observed in number of branches of the plants treated with mixed inoculation of *P. indica* and *P. fluorescens* PN026 followed by the treatment with co-cultured *P. indica* and *P. fluorescens* PN026 and the treatment of *P. indica* alone from 60^{th} day after inoculation (Table 8).

Application of co-cultured *P. indica* and *P. fluorescens* PN026 induced early flowering in the plants (31 days after transplanting) which was statistically on par with the treatment in which the mixed inoculation of *P. indica* and *P. fluorescens* PN026 was applied (34.88 days after transplanting). A delayed flowering was observed in the

plants treated with the bacterial strain *P. fluorescens* AMB8 alone. There were no significant difference in the days to fruit set among the treatments (Table 9 and 10).

Maximum number of fruits were observed in the plants treated with co-cultured *P. indica* and *P. fluorescens* PN026 (11.25) and was on par with the number of fruits obtained from the treatment of mixed inoculation of *P. indica* and *P. fluorescens* PN026 (10.25). A reduced number of fruits were recorded from the treatment co-cultured *P. indica* and *P. fluorescens* AMB8 (7.21) (Table 11).

Higher fresh fruit yield was recorded in the treatment with co-cultured *P. indica* and *P. fluorescens* PN026 (37.95 g/ plant) and the data was statistically on par with the treatments with mixed inoculation of *P. indica* and *P. fluorescens* PN026 and individual application of *P. indica* (37.78 and 34.08 respectively). Fresh fruit yield in the plants treated with co-cultured *P. indica* and *P. fluorescens* AMB8 was lower than all other treatments including the uninoculated control (Table 12).

Observations on dry weight and fresh weight of root and shoot were taken after uprooting the plants in the 80th day after transplanting. When the shoot fresh weight as well as dry weight were analyzed statistically, there were no significant difference observed among the treatments. The highest value was recorded in the treatment of mixed inoculation of *P. indica* and *P. fluorescens* PN026 (49.91g and 8.71g respectively) (Table 13).

Application of *P.indica* and *P. fluorescens* PN026 in combination resulted in the highest root fresh weight and dry weight (21.13 and 8.26 respectively) which was at par with all other treatments including *P.indica* either applied individually or combined with bacterial strains both as cocultured mixture and mixed inoculation. The plants treated with the bacterial strain *P. flurescens* PN026 alone had a reduced root dry weight as well as fresh weight (Table 14 and Plate 10).

Among all the plant growth parameters analysed, either the uninoculated control or the treatment with co-cultured *P. indica* and *P. fluorescens* AMB8 showed the lowest value. So it is clear that the co-cultured *P. indica* and *P. fluorescens* AMB8 have no additional advantage in the plant growth promotion of chilli.

Results of the plant growth promotion experiment showed that there were significant differences in plant growth parameters between the effects of the combined application of *P. indica* and *P. fluorescens* PN026, both as co-culture and as mixed inoculum and all other treatments. Among all the growth parameter analysed, the

maximum value were obtained in plants treated with either co-cultured *P. indica* or *P. fluorescens* PN026 or with a mixed inoculation of the same organisms (Plate 11).

4.4. ROOT COLONIZATION BY *Piriformospora indica*

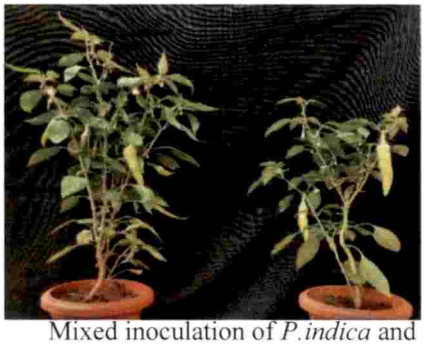
Roots of all the surviving plants treated with *Piriformospora indica* and the combinations of *Piriformospora indica* with bacterial strain both as mixed inoculum and co-cultured mixture were stained to assess the extent of root colonization by the fungal entophyte (Plate 12 and 13). The percentage root colonization of *P. indica* is given in Table 15. Plants treated with cocultured *Piriformospora indica* and *Pseudomonas fluorescens* PN026 alone showed highest root colonization with 36.39 percent followed by *P.indica* alone (35.75 percent), combination by *P. indica* with *Pseudomonas fluorescens* PN026 (35.72 percent), cocultured *Piriformospora indica* and *Pseudomonas fluorescens* AMB8 (33.62 percent) and mixed inoculation of *P.indica and Pseudomonas fluorescens* AMB8 (33.18 percent).

P. fluorescens PN026 Vs Control

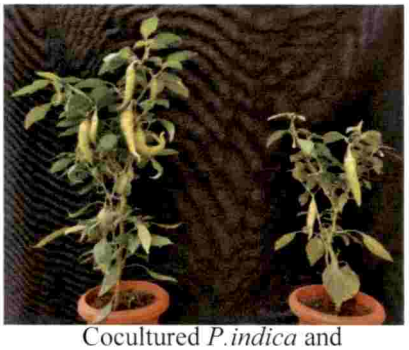
P. fluorescens AMB8 Vs control

P.indica Vs control

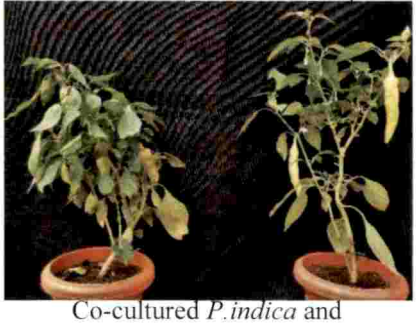
Mixed inoculation of *P.indica* and *P. fluorescens* PN026 Vs Control



Mixed inoculation of *P.indica* and *P. fluorescens* AMB8 Vs control



Cocultured *P.indica* and *P. fluorescens* AMB8 Vs control



Co-cultured *P.indica* and *P. fluorescens* PN026 Vs Control

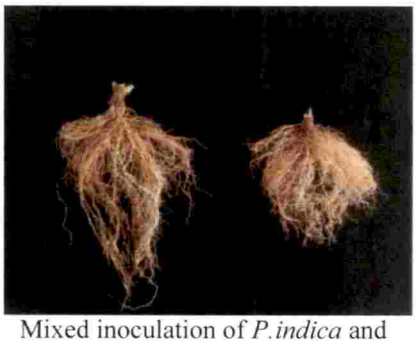
Plate 11. Assesesment of plant growth promotion by bioagents in chilli variety Vellayani Athulya

P. fluorescens PN026 Vs Control

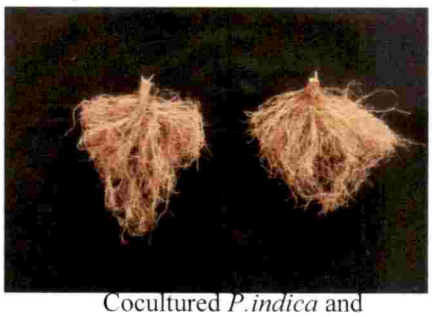
P. fluorescens AMB8 Vs control

P.indica Vs control

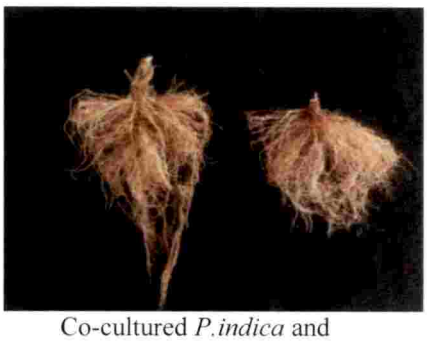
Mixed inoculation of *P.indica* and *P. fluorescens* PN026 Vs Control



Mixed inoculation of *P.indica* and *P. fluorescens* AMB8 Vs control



Cocultured *P.indica* and *P. fluorescens* AMB8 Vs control



Co-cultured *P.indica* and *P. fluorescens* PN026 Vs Control

Plate 10. Assesesment of root growth promotion by bioagents in chilli variety Vellayani Athulya

Plate 12. Chlamydospores of *P. indica* within root hairs of chilli

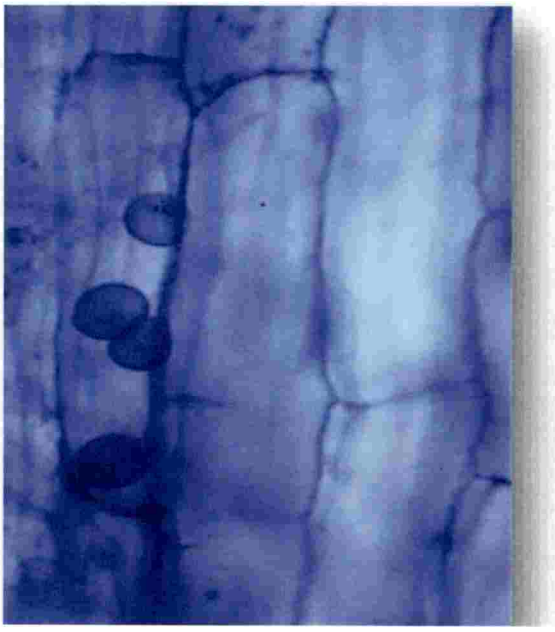


Plate 13. Colonization of *P.indica* within the root cortex cells of chilli plant

Table 12. Biometric observation on chilli treated with various bioagents on variety Vellayani Athulya after harvest (80 DAT) – fresh fruit yield (g/plant).

Treatments	Fresh fruit yield (g/plant)
Pseudomonas fluorescens **PN026**	24.54
Pseudomonas fluorescens **AMB8**	22.74
Piriformospora indica	34.08
Pseudomonas fluorescens **PN026 and** ***Piriformospora indica***	37.78
Pseudomonas fluorescens **AMB8 and** ***Piriformospora indica***	33.97
Co-cultured ***Pseudomonas fluorescens*** **PN026 and** ***Piriformospora indica***	37.95
Co-cultured ***Pseudomonas fluorescens*** **AMB8 and** ***Piriformospora indica***	21.31
Uninoculated control	21.61
SEm (±)	1.73
CD (0.05)	5.06

*Mean of three replication having five plants each

Table 13. Biometric observation on chilli treated with various bioagents on variety Vellayani Athulya after harvest (80 DAT) – Fresh weight and dry weight of shoot (g)

Treatments	Fresh shoot weight (g)	dry shoot weight (g)
Pseudomonas fluorescens PN026	45.92	7.93
Pseudomonas fluorescens AMB8	39.93	6.06
Piriformospora indica	42.23	7.16
Pseudomonas fluorescens PN026 and *Piriformospora indica*	49.91	8.71
Pseudomonas fluorescens AMB8 and *Piriformospora indica*	39.62	6.54
Co-cultured *Pseudomonas fluorescens* PN026 and *Piriformospora indica*	38.39	6.41
Co-cultured *Pseudomonas fluorescens* AMB8 and *Piriformospora indica*	37.79	5.85
Uninoculated control	32.40	6.33
SEm (±)	7.348	1.27
CD (0.05)	NS	NS

*Mean of three replication having five plants each

Table 14. Biometric observation on chilli treated with various bioagents on variety Vellayani Athulya after harvest (80 DAT) – Fresh weight and dry weight of root (g)

Treatments	Fresh root weight (g)	Dry root weight (g)
***Pseudomonas fluorescens* PN026**	12.78	3.54
***Pseudomonas fluorescens* AMB8**	15.32	3.69
Piriformospora indica	19.73	7.05
Pseudomonas fluorescens* PN026 and *Piriformospora indica	21.14	8.26
Pseudomonas fluorescens* AMB8 and *Piriformospora indica	18.31	7.25
Co-cultured *Pseudomonas fluorescens* PN026 and *Piriformospora indica*	18.12	7.45
Co-cultured *Pseudomonas fluorescens* AMB8 and *Piriformospora indica*	17.96	6.52
Uninoculated control	13.18	3.84
SEm (±)	1.92	0.71
CD (0.05)	5.63	2.08

*Mean of three replication having five plants each

Table 15. ***P. indica*** **root colonization (%) in chilli , 7 days after transplanting**

Treatments	***P.indica*** **root colonization (%)**
Piriformospora indica	35.75
Pseudomonas fluorescens **PN026 and** ***Piriformospora indica***	35.73
Pseudomonas fluorescens **AMB8 and** ***Piriformospora indica***	33.18
Co-cultured ***Pseudomonas fluorescens*** **PN026 and** ***Piriformospora indica***	36.39
Co-cultured ***Pseudomonas fluorescens*** **AMB8 and** ***Piriformospora indica***	33.63

*Mean of three replication having five plants each

Discussion

5. DISCUSSION

In modern cultivation practices, indiscriminate use of fertilizers, particularly the nitrogenous and phosphorus, has led to substantial pollution of soil, air and water. Excessive use of these chemicals exerts deleterious effects on soil microorganism, affects the fertility status of soil and also pollutes environment. Over the last few decades, the agriculture policy in India has undergone a major change through diversification and emphasis on sustainable production system.

Microbial technologies have been applied to various agricultural and environmental problems with considerable success in recent years. Biofertilizer and biopesticide containing efficient microorganisms improve plant growth in many ways compared to synthetic fertilizers, insecticides and pesticides by way of enhancing crop growth and thus help in sustainability of environment and crop productivity.

The major influences that the rhizosphere microorganisms have on plants have become important tool to guard the health of plants in ecofriendly manner. These microorganisms can effect improved plant growth and are often referred to as a plant growth promoting rhizobacteria. They are involved in various biotic activities of the soil ecosystem to make it dynamic for nutrient turn over and sustainable for crop production. In recent years considerable attention has been paid to PGPR to replace agrochemicals (fertilizers and pesticides) as they promote plant growth by a variety of mechanisms that involve soil structure formation, decomposition of organic matter, recycling of essential elements, solubilization of mineral nutrients, production of numerous plant growth regulators, degrading organic pollutants, stimulation of root growth etc. These functions are crucial for soil fertility, biocontrol of soil and seed borne plant pathogens and in promoting changes in vegetation.

Other than rhizosphere microorganisms, yet another group of plant associated microflora that has attracted research attention is the endophyte microorganisms.

Endophytes are symptomless microorganisms living inside plant that enhance host plant growth, improve nutrients uptake, reduce disease severity and enhance host tolerance to environmental stresses. Besides being highly diverse in nature, these endophytes are a novel source of bioactive secondary metabolites. Host-plants without endophyte-fungal association are devastated by the waves of extreme temperature, drought, salinity and pathogen attack. Hence, productivity is frequently compromised in such situations. These endophytes fetch higher macro- and micro-nutrients like phosphorus, sulfur, calcium, magnesium and potassium. This capability has often been considered due to the potential of these endophytes to produce various biologically active metabolites and enzymes. Among metabolites, plant hormones like GAs and auxin production is a new phenomenon in the endophytic fungi. Both GAs and auxin have been reported to play a pivotal role in plant growth, reproduction, metabolism and respone to various environmental cues. In last decade or so, it has been a known fact that these endophytic fungi, residing inside host confer abiotic stress tolerance. However, the exact mechanism is still unexplored. All these factors makes endophytes a potential tool for sustainable agriculture.

Major objective of the current study was to assess the compatibility of the root endophytic fungus *Piriformospora indica* and two *Pseudomonas fluorescens* strains and to evaluate their effect on growth promotion in chilli.Two rhizobacterial isolates were used in the present study were *P. fluorescens* PN026 and *P. fluorescens* AMB8. These isolates were obtained from previous studies conducted at the Department of Agricultural Microbiology, College of Agriculture, Vellayani and were proved to be excellent in promoting plant growth and also suppressing wide range of diseases (Nair *et al.*, 2007; Anith 2009; Nair and Anith, 2009; Varkey *et al.*, 2018).

The fungal root endophyte used in the study was *P. indica* provided by Dr. Ajit Varma, former Professor, Jawaharlal Nehru University, New Delhi and available at the Department of Agricultural Microbiology College of Agriculture, Vellayani. As reported by several workers this fungus acts as a plant growth promoter and nutrient

mobilizer in nutrient deficient soils, bio-protector against biotic and abiotic stress including root and leaf fungal pathogens, bio-regulator for plant growth development such as early flowering, enhanced seed production etc. (Oelmüller *et al.* 2009; Franken 2012; Varma *et al.* 2012).

The whole course of the research was divided into two, *in vitro* and *in vivo* experiments. As a first step to understand the microbial interaction between the endophytic fungus and the bacterial bioagents, *in vitro* evaluation of antagonism of bacteria against the endophytic fungus was performed. *In vitro* trials are preliminary screening methods, which can be used to select the putative combination for efficient consortium development (Anith *et al.*, 2003; Lemessa and Zeller, 2007). This would help to obtain the most effective combination of organisms to be used in the *in vivo* experiment. Though the laboratory conditions differ from the field conditions, it is assumed that the results obtained in the lab condition may get reflected in the field trial as well.

The *in vitro* tests performed in the current study were further divided into two; namely, direct and indirect antagonism. In direct antagonism, the microorganisms are used as such whereas in indirect antagonism the microorganisms are not directly involved but the cell free culture filtrates that may contain metabolites, enzymes and other substances that may interact with the test organisms. The indirect antagonism was checked by two methods; agar well diffusion method and paper disc diffusion method.

Dual culture plate assay was done to test the compatibility of bacterial bioagents with *P. indica* on PDA and CWA plates as both the fungal endophyte and the bacterial agents could grow well on them. Compatibility was assessed by lack of any inhibition zone whereas, the non-compatible ones would develop zone of inhibition. In the present screening on PDA plates both *Pseudomonas fluorescens* PN026 and *Pseudomonas fluorescens* AMB8 were found to be incompatible with *P. indica* in which the former one developed a reduced zone of inhibition. When screening was

done on CWA plates only the strain *Pseudomonas fluorescens* AMB8 developed zone of inhibition and the strain *Pseudomonas fluorescens* PN026 was compatable with the fungus. *P. indica* had varying reactions with different rhizobacterial isolates. When co-cultured on agar plates some of them displayed neutral response, however many displayed stimulatory to inhibitory responses (Varma *et al.,* 2012). In an experiment done by Anith *et al.* (2015) dual culture assay between *P. indica* and two *Bacillus* strains showed differential response. Zone of inhibition was larger for *B. amyloliquefaciens* whereas no antagonistic effect was seen with *B. pumilus* when the screening was done on coconut water agar medium. This implied that *P. indica* could be co-cultured with *B. pumilus*. Dual culture plate assay done by Varkey *et al* (2018) using *B. pumilus* VLY17 and *P. fluorescens* AMB8 with *P. indica* on PDA exhibited inhibition pattern and both the strains were incompatible with the endophytic fungus. In the former case however the screening was done in CWA medium. Differential inhibition pattern for the same bacterial strain on different media indicates the influence of the screening medium in determining the interaction between the microorganisms or variability among the isolates (Fig 1).

In both of these indirect methods, same trend was observed. In case of *P. fluorescens* PN026, the zone of inhibition was getting reduced rapidly and reached a negligibly small value in the third day of observation. In case of *P. fluorescens* AMB8 a higher zone of inhibition compared to *P. fluorescens* PN026 was observed and it was remaining as almost stable. It was reported that antagonistic activity of *Pseudomonas fluorescens* was tested successfully against various fungi and bacteria using these methods. Agarry (2005) tested the antagonistic activity of *Pseudomonas fluorescens* isolated from cassava rhizosphere against fungal pathogens like *Fusarium moniliforme* and *Aspergillus niger* efficiently by using the agar well diffusion method. Maji and Chakrabartty (2014) used culture filtrates of *Pseudomonas* spp. which exhibited zone of growth inhibition on *R. solanacearum.* Antifungal metabolites, antibiotics, enzymes

etc. secreted by the bacteria are present in the cultural filtrate and play a major role in fungal suppression. The antagonistic activity of various PGPR to fungal pathogens is usually related to the production of antifungal compounds and extracellular hydrolytic enzymes which are considered to be important in the lysis of fungal cell walls (Fig 2 and 3).

Co-culture experiments, involving *P.indica* and *Pseudomonas* strains using a single fermentation system were attempted in two different media; potato dextrose broth (PDB) and autoclaved coconut water (ACW). Co-culturing is a concept where two different microbial bioagents are grown together in a single fermentor system (Anith *et al.*, 2015). *P. fluorescens* strains P22:Y:05, S22:T:04 and S11:P:12 and *Enterobacter cloacae* were tested for antagonism against storage disease pathogens and the co culture experiments revealed that the co cultured bioagents outperformed the individual strains (Slininger *et al.*, 2010). Here the bacterial strains were co- cultured to get a mixed inoculum.

In the current study, co-culture experiment was carried out using *P.indica* and *Pseudomonas* strains PN026 and AMB8 in two media; PDB and ACW. The population of inoculated bacteria was assessed to find whether the bioagents are compatible to be grown together. In PDB both the bacterial strain population exhibited a declining growth pattern as same as that of the fungal free culture of bacteria in PDB. In ACW *Pseudomonas fluorescens* PN026 grew similar to that of the conventional medium King's medium B broth and when co-cultured with fungi the population build up was comparable with conventional medium and higher than that of the monoculture of the bacteria. In case of *Pseudomonas fluorescens* AMB8 similar trend was observed except that the population in co-culturing was lower than that of the monoculture of the bacteria. Co-culturing of the endophytic fungus *P. indica* and bacterial bioagents was reported for the first time by Anith *et al.*, (2018). *P. indica* takes two or more weeks

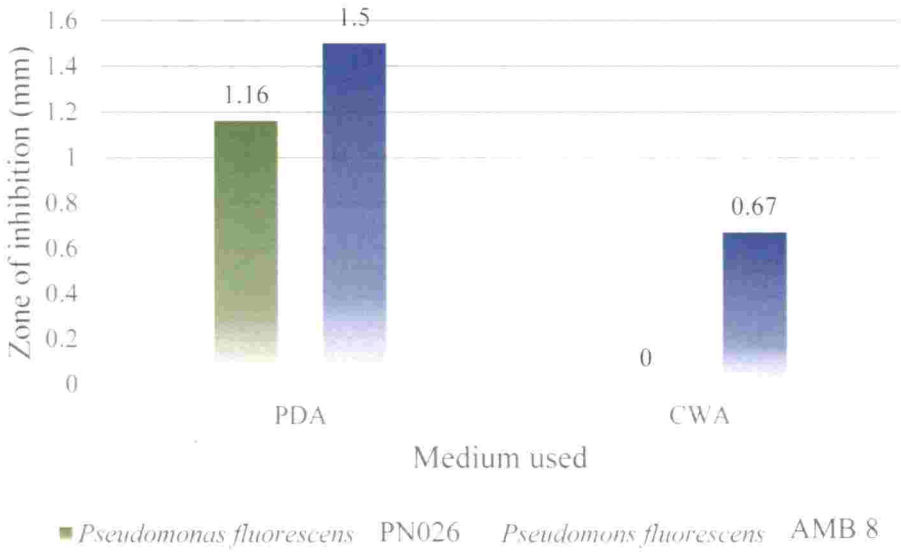


Figure 1: Mycelial growth inhibition of *P.indica* by bacterial strains on the 7th day after inoculation in dual culture plate assay

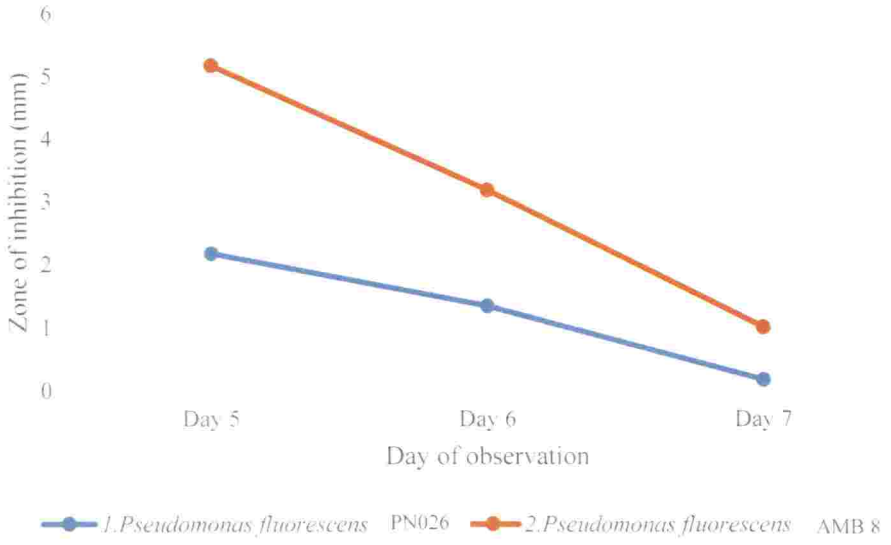


Figure 2: Mycelial growth inhibition of *P.indica* by bacterial strains in agar well diffusion technique

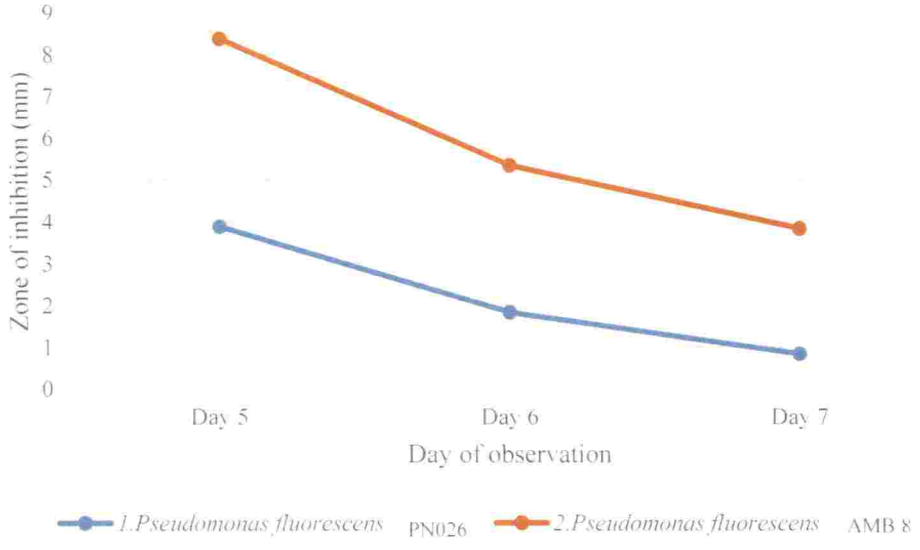


Figure 3: Mycelial growth inhibition of *P.indica* by bacterial strains in paper disc diffusion technique

to grow well in broth culture. It was earlier found out that the broth in which the fungal growth occurs would further support the growth of compatible bacterial isolates when inoculated. In a study conducted by Anith (2009) PGPR strains, *Pseudomonas* sp PN026R and *B. pumilus* SE34 were found to multiply well in coconut water utilizing it as a sole source of nutrients. When they were grown in CW collected in conical flask as well as within intact coconut, the growth pattern was comparable to that in conventional liquid medium When *B. pumilus* and *P. Indica* were co-cultured, the bacterial bioagent showed similar growth rate as that of its monoculture (Anith *et al.*, 2015). Similar to the result obtained in the present study, it was reported that co-culturing of *Bacillus pumilis* with *P. indica* in PDB resulted in a decline in population buildup of bacteria (Anith *et al.,* 2015) (Fig 4 and 5).

Once the *in vitro* tests with respect to the antagonistic interactions are understood, the next step is to validate the same under *in vivo* conditions either in a green house or in the open field. In the present study, pot culture experiments were performed under open field conditions using unsterile soil system. In the nursery sterile vermiculate was used as it would encourage better root growth and assured colonization by the applied bioagents as competition from other microorganisms in the planting medium is completely avoided. Bio priming of seeds in the nurseries may have added advantage over the field inoculation with the bio agents.

Production of good quality seedlings is a major aspect in transplanted vegetables. Healthy and disease free transplants ensure high rate of establishment and realization of high potential yield. Raising seedling of vegetables using plug trays (pro-trays) allows near perfect crop stands by assuring uniform physiological stage of the seedlings during transplanting (Vavrina, 1998). Plug tray seedlings enable quicker re-establishment due to less transplanting shock. Plug tray transplants are commercially used in the production system of chilli.

Application of biological agents at the nursery stage is advantageous as they get established in the transplants and the bio primed plants are carried to the field effectively. Biological amendment with inoculants in transplant production of vegetables has been reported earlier (Gagne *et al.*, 1993; Nemec *et al.*, 1996; Kokalis-Burelle *et al.*, 2002; Russo, 2006; Russo and Perkins-Veazie, 2010). In the present study, plant growth promotion by bacterial bioagent, fungal endophyte, it,s mixed inoculation and co-cultured inoculum was evaluated in the variety Vellayani Athulya in the pot culture.

Statistical analysis of various growth parameters revealed that there was a significant enhancement in the growth of plants treated with combined application of *P. indica* and *P. fluorescens* PN026, both as coculture and mixed inoculum. With respect to plant height there was no significant difference among the treatment (Fig 6). In a study conducted in tomato, similar result was observed when *P. indica* and *Bacillus pumilis* were applied individually and as combined form (both mixed inoculation and co-cultured inoculation). There was no significant difference in the plant height among the treatments (Anith *et al.,* 2015). However a significant increase in number of leaves were observed in the tomato seedlings treated with either the application of mixed inoculation or co-cultured suspension of the bioagents. It is also comparable with the results obtained from the present study in which highest number of leaves were recorded in the plants treated either with mixed or co-cultured combination of *P. indica* and *P. fluorescens* PN026 (Fig 7)

A significantly increasing trend was observed in number of branches of the plants treated with mixed inoculation of *P. indica* and *P. fluorescens* PN026 followed by the treatment with co-cultured *P. indica* and *P. fluorescens* PN026 and the treatment of *P. indica* alone from 60th day after inoculation. The endophytic fungus has been reported to improve the uptake of nitrogen by plants through the enhanced expression of nitrate

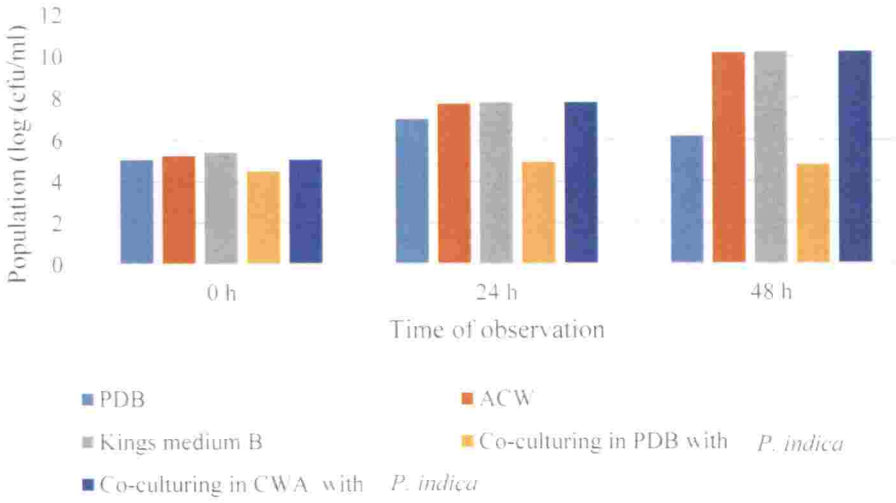


Figure 4: Population buildup of *Pseudomonas fluorescens* AMB8 (cfu ml^{-1}) in different media and cultural condition

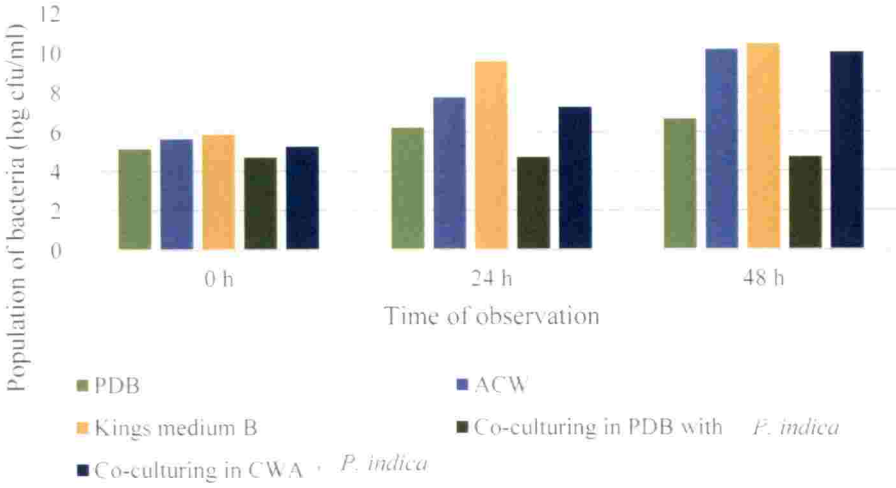


Figure 5: Population buildup of *Pseudomonas fluorescens* PN026 (cfu ml^{-1}) in different media and cultural condition

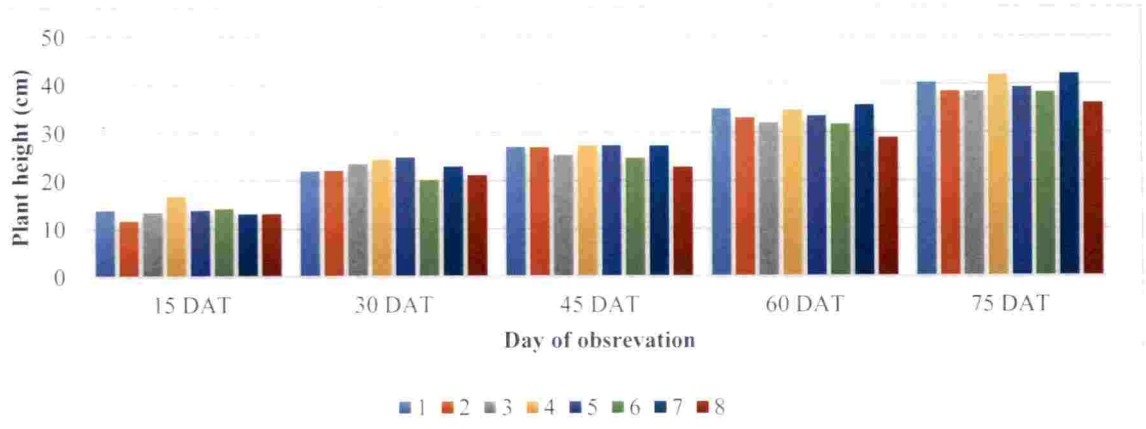


Figure 6: Plant height of the chilli variety Vellayani Athulya taken in 15 days interval on inoculation with bioagents

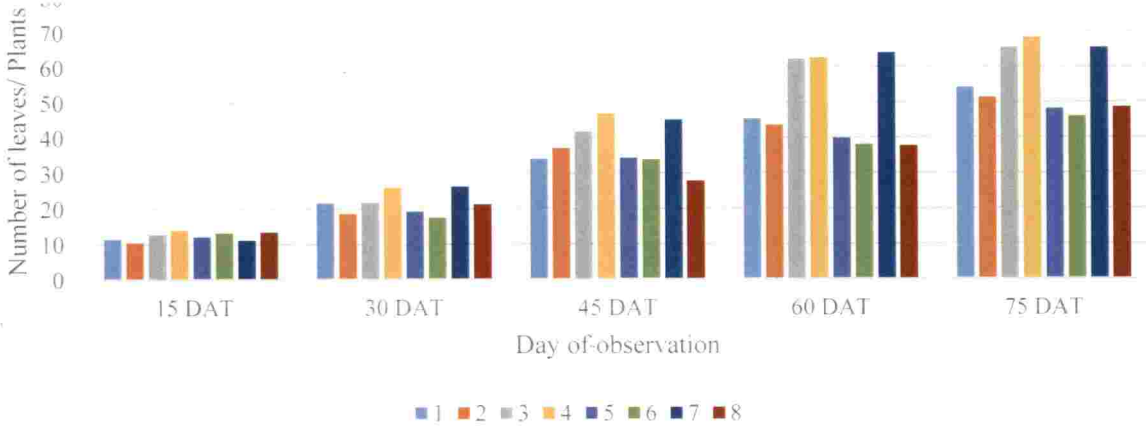


Figure 7: Number of leaves of the chilli variety Vellayani Athulya taken in 15 days interval on inoculation with bioagents

1- *P. fluorescens* PN026; 2- *P. fluorescens* AMB8; 3- *P. indica;* 4- Mixed inoculation of *P. fluorescens* PN026 and *P. indica;* 5- Mixed inoculation of *P. fluorescens* AMB8 and *P. indica*; 6-Uninoculated control; 7- Co-cultured *P. fluorescens* PN026 and *P. indica* ; 8- - Co-cultured *P. fluorescens* AMB8 and *P. indica*

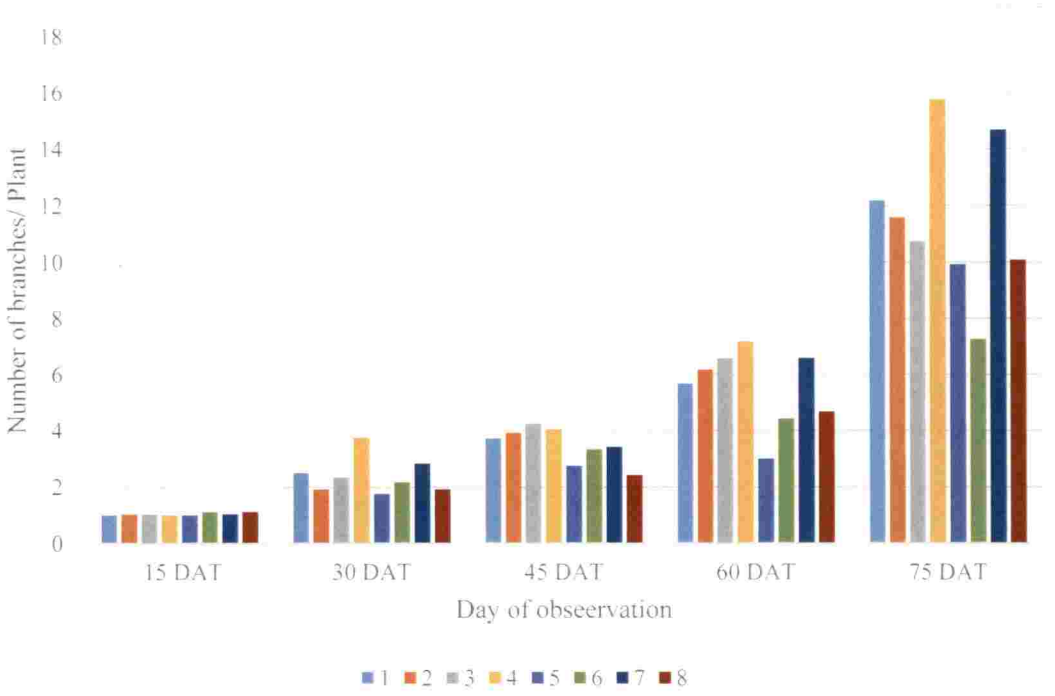


Figure 8: Number of branches of the chilli variety Vellayani Athulya taken in 15 days interval on inoculation with bioagents

1- *P. fluorescens* PN026; 2- *P. fluorescens* AMB8; 3- *P. indica;* 4- Mixed inoculation of *P. fluorescens* PN026 and *P. indica;* 5- Mixed inoculation of *P. fluorescens* AMB8 and *P. indica*; 6-Uninoculated control; 7- Co-cultured *P. fluorescens* PN026 and *P. indica* ; 8- - Co-cultured *P. fluorescens* AMB8 and *P. indica*

reductase in plant roots (Sherameti *et al.,* 2005). Various strains of *P. fluorescens* have been reported as effective as that of 100 percent fertilizer application (Gamez *et al.,* 2019). The improved vegetative growth can be attributed to the synergetic effect by combining the organisms in mixed application which enhanced the growth promotion capacity of *P. indica* and *P. fluorescens.* Number of branches were significantly higher only from 60 days after transplanting. It may be due to the reprogramming of root exudation pattern of the host by endophytic fungus and thus increasing the population of PGPR in the rhizosphere of chilli as reported by Saxena *et al.* (2015).

Application of co-cultured *P. indica* and *P. fluorescens* PN026 induced early flowering in the plants followed by the plants treated with mixed inoculation of *P. indica* and *P. fluorescens* PN026. There is no significant different in the days to fruit set among the treatmemts. Earliness in flowering in *P. indica* applied black pepper plants has been reported by Anith *et al.* (2018). The medicinal plants *Spilanthes calva* and *Withania somnifera* were inoculated with *Piriformospora indica* and it was observed that number of inflorescences and flowers and seed production were all enhanced in the presence of the fungus (Rai *et al.,* 2014). A study conducted in the medicinal plant, *Coleus forskohlii, P. indica* colonized plants flowered at least 7 day earlier and more vigorously than the non-colonized plants. It was suggested that the increase in flower production may be caused by an increase in plant nutrient (especially K^+ and P) uptake by the fungal endophyte, in combination with a possible hormonal effect. Hormones, such as gibberellins that induce the bud production could be transported in faster rates due to higher levels of K^+ in the plant and phosphorus have a great impact on bud formation and development (Das *et al.,* 2012) (Fig 9 and 10).

Teles *et al.* (2012) reported that co-culturing bacterial-fungal interactions give rise to enhanced effects of biosynthesis compared to their monocultures. Thus the early flowering in the treatment with co-cultured *P. indica* and *P. fluorescens* PN026 may

be due to the enhancement of mechanism of *P. indica* to induce early flowering by *P. fluorescens* PN026 in co-culturing system (Fig 9 and 10).

Maximum number of fruits and highest yield was recorded in the co-cultured *P. indica* and *P. fluorescens* PN026 treated plants. A consortium containing *P. indica* and two Pseudomonad strains (R62 and R81) when applied to mung bean, an increased number of pods harvested were noted in the consortial treatment compared to the control (Kumar *et al.*, 2012). The result from this present study is comparable with the result that reported above (Fig 11 and 12).

The highest values were recorded in the treatment of mixed inoculation of *P. indica* and *P. fluorescens* PN026 in the case of shoot fresh weight as well as dry weight (Fig 13) These beneficial effect in plant growth may be due the additive effect of improved nutrient supply by the endophyte as same as that of arbuscular mycorrhizal symbiosis and the ability of the *Pseudomonas* strains to promote the plant growth as reported by Saxena *et al.* (2015).

Application of *P.indica* and *P. fluorescens* PN026 in combination resulted in the highest root fresh weight and dry weight which was at par with all other treatments including *P.indica* either applied individually or combined with bacterial strains both as co-cultured mixture and mixed inoculation (Fig 14). The possible reason may be due to the ability of *P. indica* to enhance the root growth and number of adventitious root as reported in many studies. Sirrenberg *et al.* (2007) attributed the ability of this fungus to enhance root growth promotion to the production of auxin inside plant after the endophyte get established. Justice *et al.* (2018) reported potential enhancement of adventitious root formation as well as increase in root weight in the flowering plants like crossandra, dahlia and poinsettia when the cuttings were planted in rooting medium amended with *P. indica*. Tomato seedlings treated with co-cultured *P.indica* and *Bacillus pumilis* recorded highest root fresh weight and dry weight compared to the

treatments with mixed inoculation and individual application of the bioagents (Anith *et al.,* 2015).

Plants treated with co-cultured *Piriformospora indica* and *Pseudomonas fluorescens* PN026 showed highest root colonization followed by *P. indica* alone, combination by *P. indica* with *Pseudomonas fluorescens* PN026, co-cultured *Piriformospora indica* and *Pseudomonas fluorescens* AMB8 and mixed inoculation of *P.indica and Pseudomonas fluorescens* AMB8 (Fig 15). Anith *et al.* (2015) also reported that co-cultured *P. indica* and *Bacillus pumilis* showed increased endophytic colonization in root of tomato compared to its mixed inoculation and individual application. In a study conducted by Jayasinghearchchi and Seneviratne (2010) reported that an endophytic fungus *Pleurotus ostreatus* co-cultured with *Pseudomonas fluorescens* improved the endophyte colonization of tomato through biofilm formation. The same reason can be proposed for the enhanced root colonization by the co-cultured *Piriformospora indica* and *Pseudomonas fluorescens* PN026 in chilli.

Among all the parameter analysed, either the uninoculated control or the treatment with co-cultured *P. indica* and *P. fluorescens* AMB8 showed the lowest value and it is evident that this combination had no additional advantage in the plant growth promotion of chilli. The possible reason for this may be the antagonistic effect showed by the bacterial strain against the endophytic fungus. Even though the fungal colonization percentage in the roots of plants treated with co-cultured *P. indica* and *P. fluorescens* AMB8 was comparable with all other treatments, the growth promotion by the bioagent was not visible in the plants.

Results of the plant growth promotion experiment showed that there were significant differences in plant growth parameters between the effects of the combined application of *P. indica* and *P. fluorescens* PN026, both as co-culture and as mixed inoculum and all other treatments. Root colonization by *P. indica* in plants positively influence plant growth as well as improves defensive capacity against fungal and viral

pathogens (Fakhro et al. 2010).Strains of *P. fluorescens* are reported to have plant growth promotional effects and induction of systemic resistance (ISR) (Linu *et al.,* 2019). Combining the fungal and rhizobacterial biological agents, therefore, would have additional benefits provided to the chilli plants than when applied singly. Results of the current investigation suggest that mixed inoculation and inoculation with the co-culture of *P. indica* and *P. fluorescens* PN026 are more efficient than single inoculation of the biological agents for improving plant growth in chilli. Previous reports involving other crops also supported the idea of co-inoculation of *P. indica* with beneficial bacteriata achieve greater plant growth (Meena *et al*., 2010; Sarma *et al*., 2011; Kumar *et al*. 2012; Anith *et al.,* 2015).

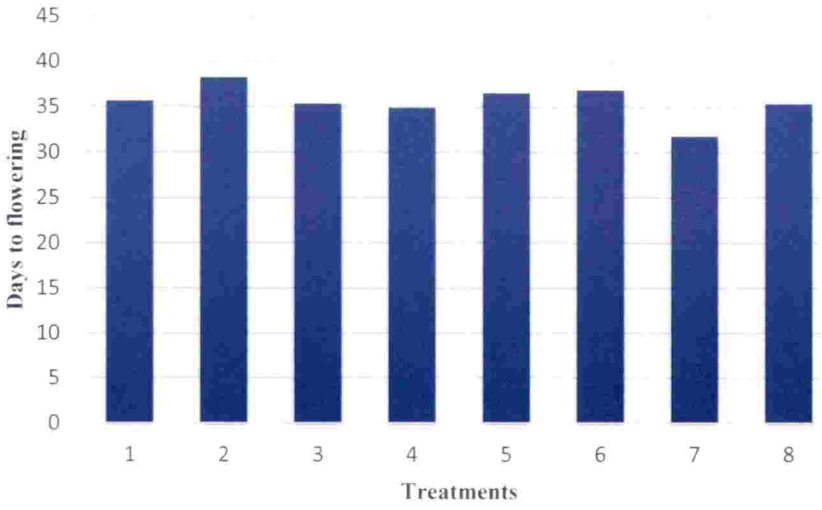


Figure9: Days to flowering of the chilli variety Vellayani Athulya on inoculation with bioagents

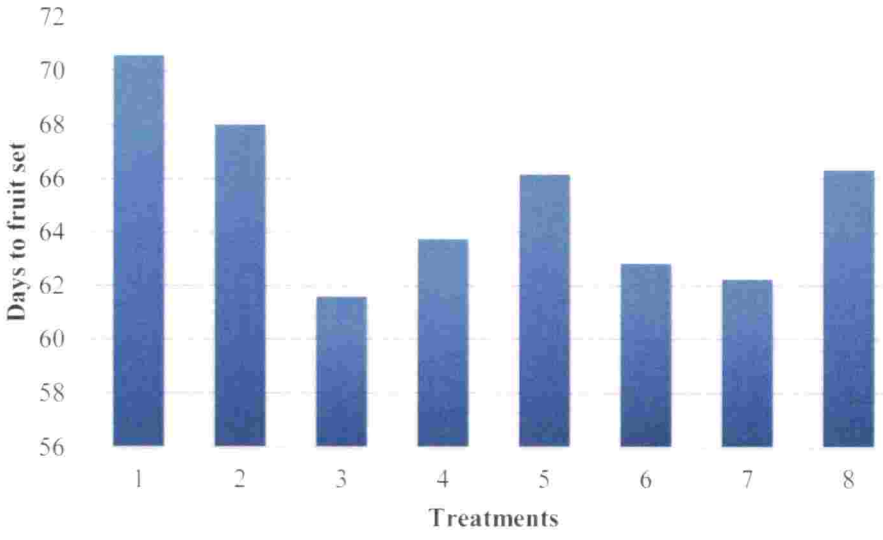


Figure10: Days to fruit set of the chilli variety Vellayani on inoculation with bioagents

1- *P. fluorescens* PN026; 2- *P. fluorescens* AMB8; 3- *P. indica;* 4- Mixed inoculation of *P. fluorescens* PN026 and *P. indica;* 5- Mixed inoculation of *P. fluorescens* AMB8 and *P. indica*; 6-Uninoculated control; 7- Co-cultured *P. fluorescens* PN026 and *P. indica* ; 8- - Co-cultured *P. fluorescens* AMB8 and *P. indica*

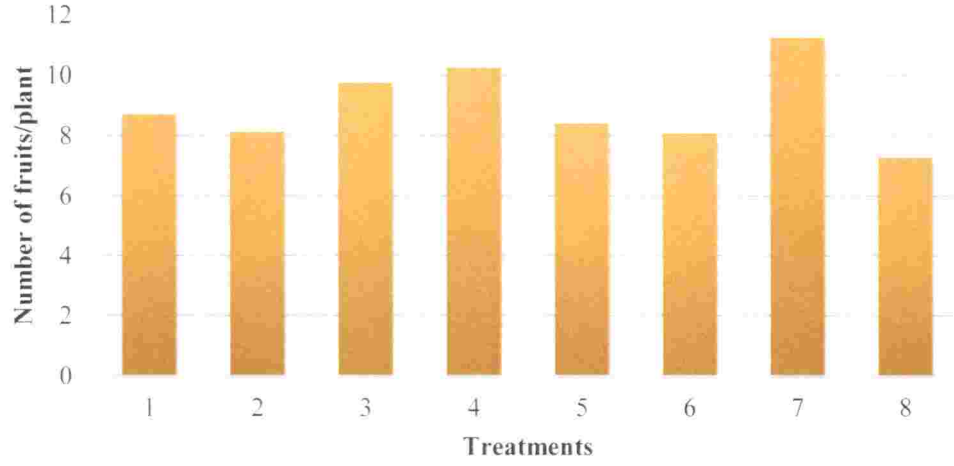


Figure 11: Number of fruits per plant of the chilli variety Vellayani Athulya taken in 80 days after transplanting on inoculation with bioagents

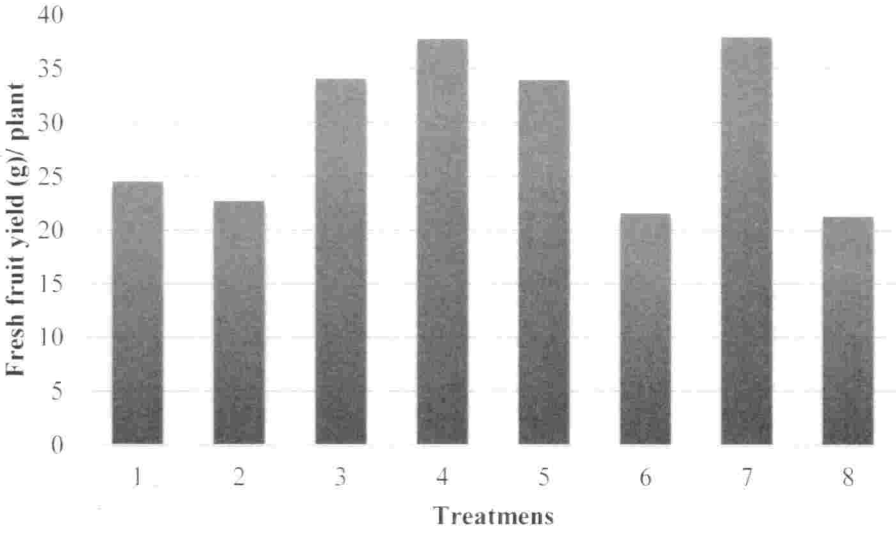


Figure 12: Fresh fruit yield of the chilli variety Vellayani Athulya taken in 80 days after transplanting on inoculation with bioagents

1- *P. fluorescens* PN026; 2- *P. fluorescens* AMB8; 3- *P. indica;* 4- Mixed inoculation of *P. fluorescens* PN026 and *P. indica;* 5- Mixed inoculation of *P. fluorescens* AMB8 and *P. indica*; 6-Uninoculated control; 7- Co-cultured *P. fluorescens* PN026 and *P. indica* ; 8- - Co-cultured *P. fluorescens* AMB8 and *P. indica*

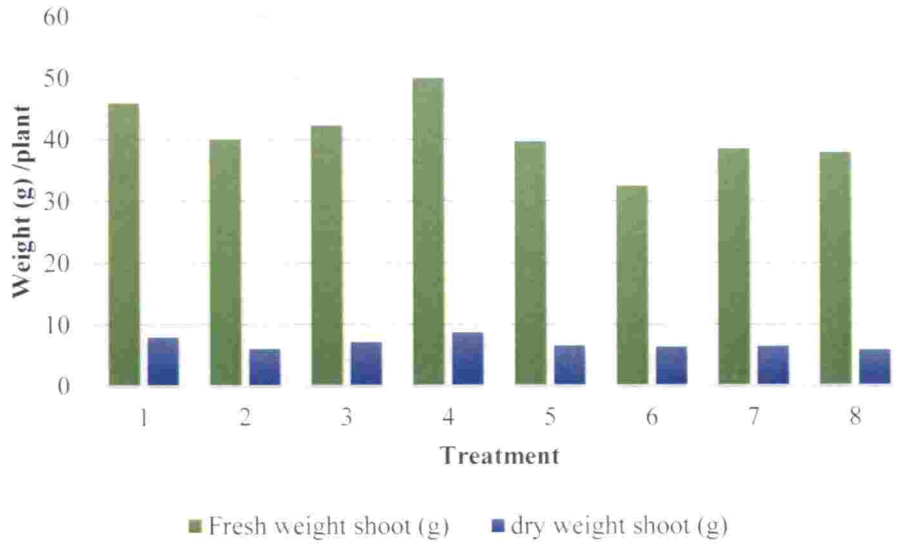


Figure 13: Fresh weight and dry weight of shoot of the chilli variety Vellayani Athulya taken in 80 days after transplanting on inoculation with bioagents

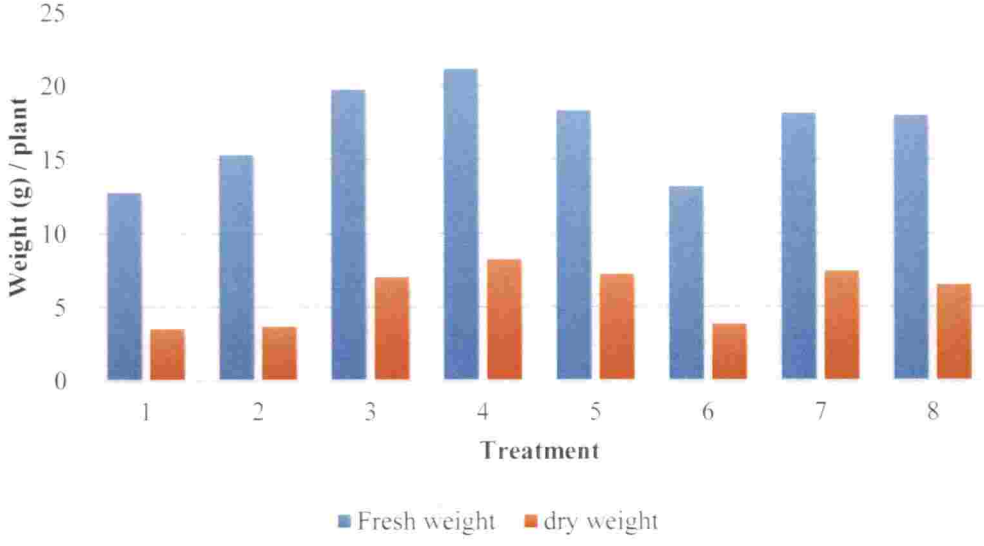


Figure 14: Fresh weight and dry weight of root of the chili variety Vellayani Athulya taken in 80 days after transplanting inoculation with bioagents

1- *P. fluorescens* PN026; 2- *P. fluorescens* AMB8; 3- *P. indica;* 4- Mixed inoculation of *P. fluorescens* PN026 and *P. indica;* 5- Mixed inoculation of *P. fluorescens* AMB8 and *P. indica*; 6-Uninoculated control; 7- Co-cultured *P. fluorescens* PN026 and *P. indica* ; 8- - Co-cultured *P. fluorescens* AMB8 and *P. indica*

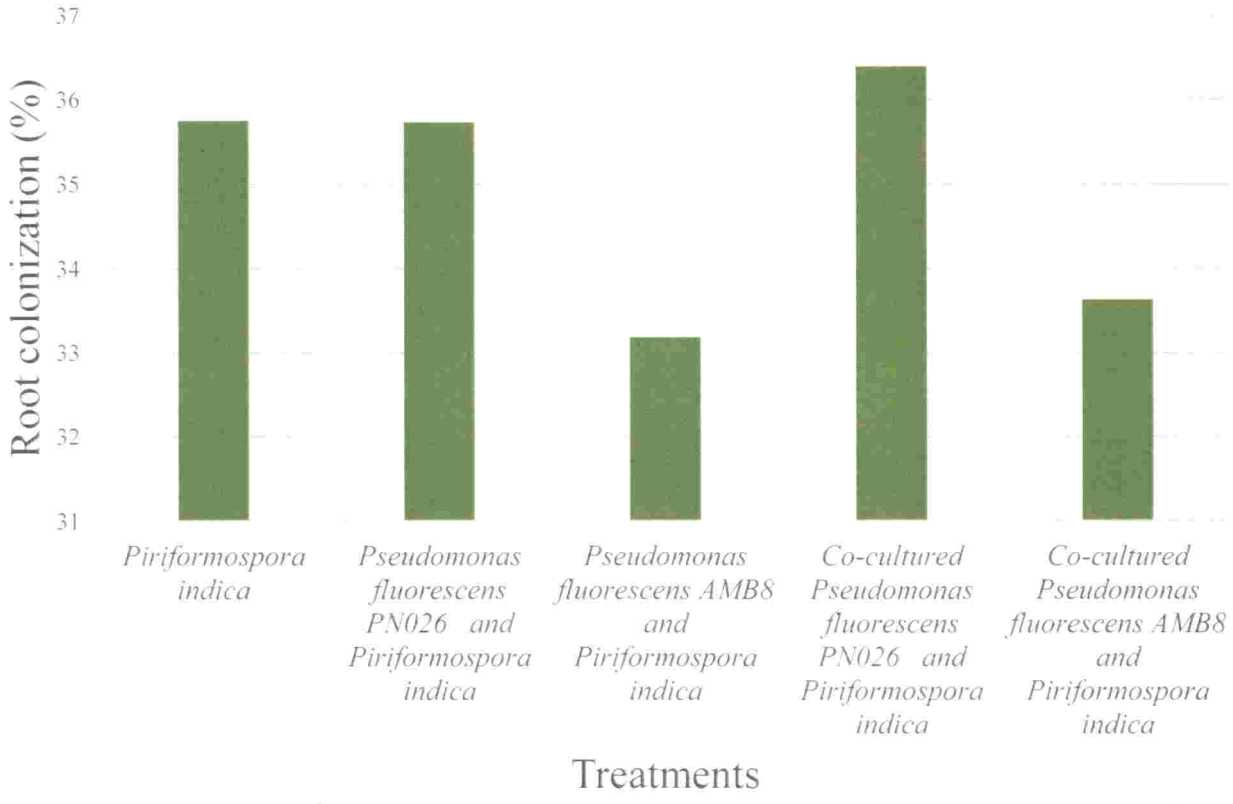


Figure 15: Root colonization in percentage of the chilli variety Vellayani n inoculation with bioagents

Summary

6. SUMMARY

The investigation entitled "Growth promotion in chilli (*Capsicum annuum* L.) on inoculation with *Pseudomonas fluorescens* and *Piriformospora indica*"was conducted during 2017-2019 at Department of Agricultural microbiology, College of Agriculture, Vellayani. Major objective of the study was to assess the compatibility of the root endophytic fungus *Piriformospora indica* and two *Pseudomonas fluorescens* strains and to evaluate their effect on growth promotion in chilli.

Biofertilizer is a potential alternative for chemical fertilizer. Extensive use of chemical fertilizer is hazardous to environment and not economical to farmers. So scientists around the world has recognised the importance of various bioagents as a plant growth promoting tool and numerous studies are being conducted to explore the full potential of the microorganisms as an alternative for agrochemicals. Different bioagents like rhizosphere microorganisms, AMF, endophytes etc. are gaining importance nowadays. A consortium of beneficial microorganisms improves the efficiency of a bio-inoculant formulation, by improving the consistency of its performance. This study consisted of use of *Pseudomonas fluorescens* strains, a potential PGPR and axenially cultivable root endophytic fungi *Piriformospora indica* and its combined effect in growth promotion in chilli. A novel co-culture method of the bioagents using naturally available coconut water is also described in this study.

The whole course of the research was divided into two, *in vitro* and *in vivo* experiments. As a first step to understand the microbial interaction between the endophytic fungi and the bacterial bioagents, *in vitro* evaluation of antagonism of bacteria against the endophytic fungi was performed. The *in vitro* tests performed in the current study were further divided into two namely; direct and indirect antagonism. In direct antagonism, the microorganisms are used as such whereas in indirect antagonism the microorganisms are not directly involved but the cell free culture

filtrates that may contain metabolites, enzymes and other substances mediate the suppression of the endophytic fungi.

In vitro interaction of bacterial bioagents and *Piriformospora indica* using dual culture plate assay was done to assess the compatibility between them. The zone of inhibition was measured in 5th and 7th day after the bacterial inoculation. In PDA plates both *Pseudomonas fluorescens* PN026 and *Pseudomonas fluorescens* AMB8 inhibited the growth of *Piriformospora indica* with alone of inhibition of 1.16mm and 1.50mm respectively in 7th day after bacterial inoculation. In CWA plates *P. fluorescens* PN026 had no inhibition on *P. indica* and *P. fluorescens* AMB8 had a reduced inhibition of 0.67mm compared to PDA plates.

In agar well diffusion method, the culture filtrates of both bacterial strains exhibited antagonism against the fungus *Piriformospora indica*. In 7th day after bacterial inoculation, zone of inhibition was the higher in *P. fluorescens* AMB8 (0.84mm) than that of *P. fluorescens* PN026 (0.17 mm).

In disc diffusion method the discs impregnated with culture filtrate of *P. fluorescens* strains exhibited antagonistic activity against *Piriformospora indica* by producing inhibition zone. Zone of inhibition was maximum for *P. fluorescens* AMB8 (3 mm) and *P. fluorescens* PN026 showed zone of inhibition of 0.83 mm in 7th day after bacterial inoculation.

Co-culturing of the bacterial bioagents with the endophytic fungus *Piriformospora indica* showed varying levels of population buildup of the bacteria after 24 h and 48 h of incubation. When 10 day-old cultures of the fungus in ACW and PDB were inoculated with the bacterial strains, the former medium supported the growth of the bacteria similarly to fungus free culture and King's medium B broth. Both monoculture and co-culture in ACW resulted in achieving a population of 10^{10} cfu/ml from an initial inoculum of 10^5 cfu/ml. On the other hand co-cultivation in PDB led to a decline in bacterial population.

Once the *in vitro* tests with respect to the antagonistic interactions are understood, the next step is to validate the same under *in vivo* conditions either in a green house or in the open field. Here in the present study, pot culture experiments were performed under open field conditions using unsterile soil system. Growth parameters like plant height, number of leaves and number of branches were measured in fifteen days interval after transplanting. With respect to plant height there was no significant difference among the treatments. Maximum values were obtained in the treatment of mixed inoculation of *P. indica* and *P. fluorescens* PN026. 60 days after transplanting onwards maximum value was recorded in the treatment of co- cultured *P. indica* and *P. fluorescens* PN026.

Highest number of leaves were recorded in the plants treated either with mixed or co-cultured combination of *P. indica* and *P. fluorescens* PN026. In the observation taken in 45th day and 75th day after transplanting higher number of leaves (46.79 and 68.45 respectively) were recorded in the treatment with mixed inoculation of *P. indica* and *P. fluorescens* PN026 and the data is statistically on par with those treated with co-cultured *P. indica* and *P. fluorescens* PN026 and *P.indica* alone. In the observation taken in 60th day after transplanting, highest number of leaves (64.08) were observed in the treatment with co-cultured *P. indica* and *P. fluorescens* PN026.

A significantly increasing trend was observed in number of branches of the plants treated with mixed inoculation of *P. indica* and *P. fluorescens* PN026 followed by the treatment with co-cultured *P. indica* and *P. fluorescens* PN026 and the treatment of *P. indica* alone from 60th day after inoculation.

Application of co-cultured *P. indica* and *P. fluorescens* PN026 induced early flowering in the plants (31 days after transplanting). The data is statistically on par with the treatment in which the mixed inoculation of *P. indica* and *P. fluorescens* PN026 was applied (34.88 days after transplanting). A delayed flowering was observed in the

plants treated with the bacterial strain *P. fluorescens* AMB8 alone. There were no significant difference in the days to fruit set among the treatments.

The maximum number of fruits were observed in the plants treated with co-cultured *P. indica* and *P. fluorescens* PN026 (11.25) and it was on par with the number of fruits obtained from the treatment of mixed inoculation of *P. indica* and *P. fluorescens* PN026 (10.25). A reduced number of fruits were recorded from the treatment co-cultured *P. indica* and *P. fluorescens* AMB8 (7.21).

A higher fresh fruit yield was recorded in the treatment with co-cultured *P. indica* and *P. fluorescens* PN026 (37.95 g/ plants) and the data is statistically on par with the treatments with mixed inoculation of *P. indica* and *P. fluorescens* PN026 and individual application of *P. indica* (37.78 and 34.08 respectively). Fresh fruit yield in the plants treated with co-cultured *P. indica* and *P. fluorescens* AMB8 was lower than all other treatments including the uninoculated control.

Observations in dry weight and fresh weight of root and shoot were taken after uprooting the plants in the 80th day after transplanting. When the shoot fresh weight as well as dry weight were analyzed statistically, there were no significant difference observed among the treatments. The highest value was recorded in the treatment of mixed inoculation of *P. indica* and *P. fluorescens* PN026.

Application of *P.indica* and *P. fluorescens* PN026 in combination resulted in the highest root fresh weight and dry weight (21.13 and 8.26 respectively) which was at par with all other treatments including *P.indica* either applied individually or combined with bacterial strains both as cocultured mixture and mixed inoculation. The plants treated with the bacterial strain *P. flurescens* PN026 alone had a reduced root dry weight as well as fresh weight.

Among all the parameter analysed, either the uninoculated control or the treatment with co-cultured *P. indica* and *P. fluorescens* AMB8 showed the lowest

value. So it is clear that the co-cultured *P. indica* and *P. fluorescens* AMB8 have no additional advantage in the plant growth promotion of chilli.

Results of the plant growth promotion experiment showed that there were significant differences in plant growth parameters between the effects of the combined application of *P. indica* and *P. fluorescens* PN026, both as co-culture and as mixed inoculum and all other treatments. Among all the growth parameter analysed, the maximum value were obtained in plants treated with either co-cultured *P. indica* or *P. fluorescens* PN026 or with a mixed inoculation of the same organisms.

Roots of all the plants treated with *Piriformospora indica* and the combinations of *Piriformospora indica* with bacterial strain both as mixed inoculum and co-cultured mixture were stained to assess the extent of root colonization by the fungal entophyte. Plants treated with cocultured *Piriformospora indica* and *Pseudomonas fluorescens* PN026 alone showed highest root colonization with 36.39 percent followed by *P.indica* alone (35.75 percent), combination by *P. indica* with *Pseudomonas fluorescens* PN026 (35.72 percent), cocultured *Piriformospora indica* and *Pseudomonas fluorescens* AMB8 (33.62 percent) and mixed inoculation of *P.indica and Pseudomonas fluorescens* AMB8 (33.18 percent).

Results of the current investigation suggest that mixed inoculation and inoculation with the co-culture of *P. indica* and *P. fluorescens* PN026, which are compatible each other *in vitro* studies is more efficient than single inoculation of the biological agents for improving plant growth in chilli.

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Appendices

APPENDIX- 1

COMPOSITION OF MEDIA USED

1. King's medium B

Peptone	-	20 g
K_2HPO_4	-	1.5 g
$MgSO_4$	-	1.5 g
Glycerol	-	10 ml
Agar	-	20 g
Distilled water	-	1000 mL

Peptone, K_2HPO_4 and $MgSO_4$ were dissolved in distilled water containing glycerol. Agar-agar was added into this mixture and autoclaved at 15lbs pressure and 121°C for 15 minutes.

2. Potato Dextrose Agar

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

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

GROWTH PROMOTION IN CHILLI (*Capsicum annuum* L.) ON INOCULATION WITH *Pseudomonas fluorescens* AND *Piriformospora indica*

by

Nandana. M. S.

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ABSTRACT

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COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695 522

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123

ABSTRACT

The study entitled "Growth promotion in chilli (*Capsicum annuum* L.) on inoculation with *Pseudomonas fluorescens* and *Piriformospora indica*" was undertaken during 2017-2019, in the Department of Agricultural Microbiology, College of Agriculture, Vellayani, Thiruvananthapuram, with the objective to assess the compatibility of the root endophytic fungus *Piriformospora indica* and two *Pseudomonas fluorescens* strains, and to evaluate their effect on growth promotion in chilli variety Vellayani Athulya. The Pseudomonad strains used were *Pseudomonas fluorescens* PN026 and *Pseudomonas fluorescens* AMB8.

Experiments comprised both *in vitro* and *in vivo* studies. For *in vitro* study a dual culture plate assay was done in potato dextrose agar (PDA) and coconut water agar (CWA) with the fungal and bacterial endophytes to evaluate the direct antagonism. Both *Pseudomonas fluorescens* PN026 and *Pseudomonas fluorescens* AMB8 showed antagonism to the root endophyte *Piriformospora indica* in PDA whereas in CWA, *Pseudomonas fluorescens* PN026 did not show any antagonistic effect and *Pseudomonas fluorescens* AMB8 showed a reduced antagonism to *Piriformospora indica* compaired to that in PDA.

Indirect antagonism was evaluated through agar well diffusion method and paper disc diffusion method using culture filtrate of the bacterial strains and the culture filtrate from both the bacterial strains showed antagonism against *Piriformospora indica* in which the maximum zone of inhibition was observed in culture filtrate of *Pseudomonas fluorescens* AMB8.

A Co-culture experiment involving *P.indica* and *Pseudomonas* strains using a single fermentation system was attempted in two different media; potato dextrose broth (PDB) and autoclaved coconut water (ACW). The flasks were incubated under agitation for 48 h and the population of the bacteria was determined at 24 h intervals by dilution plating in Kings B agar medium and it was observed that, when 10 day old cultures of the fungus in ACW and PDB were inoculated with the bacteria, ACW supported the growth of the bacteria similarly

to fungus free ACW and KB medium. Co-cultivation in PDB led to a decline in bacterial population and the autoclaved coconut water can be suggested as a better medium for co-culturing of *P. indica* and *Pseudomonas fluorescens* strains.

A pot culture experiment was undertaken to study the effect of the different treatments on growth promotion of chilli. The experiment was laid out in CRD with six treatments and three replications and observation was taken in 15 days. The treatments comprised fungal and bacterial endophytes along with combinations of both fungal and bacterial endophytes and an uninoculated control. Different parameters like plant height, number of leaves, number of branches, number of fruits/plant, fresh fruit yield, fresh shoot weight, dry shoot weight, fresh root weight, dry root weight, days to flowering, days to fruit set and percentage root colonisation by *Piriformospora indica* were evaluated.

Maximum plant height was recorded in the treatment with mixed inoculation of *Piriformospora indica* and *Pseudomonas fluorescens* PN026 without any significance in statistical data. A significantly increasing trend was observed in number of leaves with mixed inoculation of *Piriformospora indica* and *Pseudomonas fluorescens* PN026 from 45th day after transplanting and it was statistically on par with the treatment consisting of *Piriformospora indica* alone. Number of branches were found to be higher with mixed inoculation of *Pirifomospora indica* and *Pseudomonas fluorescens* PN026. There was no significant difference in the fresh weight and dry weight of shoot, whereas the fresh and dry weight of root (21.13 g and 8.26g respectively) were significantly higher in the plants treated with *P. indica* along with *Pseudomonas fluorescens* PN026. Number of fruits per plant (10.25/plant) and fresh fruit yield per plant (37.95g/plant) were recorded significantly higher with mixed inoculation of *Piriformospora indica* and *Pseudomonas fluorescens* PN026.

The *in vivo* study disclosed that plants treated with *Piriformospora indica* along with *Pseudomonas fluorescens* PN026 were found to perform better than all other treatments. The mixed inoculation of *Pseudomonas fluorescens* AMB8 and

P. indica had no additional advantage in plant growth in chilli. *Piriformospora indica* was able to successfully colonize in the plant roots applied with the bacterial endophyte.

The present study revealed that plant growth promoting rhizobacteria, *Pseudomonas fluorescens* PN026 can be used along with *Piriformospora indica,* the root endophyte, for enhancing plant growth in chilli.