MANAGEMENT OF BACTERIAL WILT DISEASE OF TOMATO BY THE ROOT ENDOPHYTIC FUNGUS *Piriformospora indica*, RHIZOBACTERIA AND BACTERIAL ENDOPHYTES

by

ATHIRA, S.

(2016-11-128)

THESIS

Submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM - 695 522

KERALA, INDIA

2018

DECLARATION

I, hereby declare that this thesis entitled **"MANAGEMENT OF BACTERIAL WILT DISEASE OF TOMATO BY THE ROOT ENDOPHYTIC FUNGUS *Piriformospora indica*, RHIZOBACTERIA AND BACTERIAL ENDOPHYTES"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Vellayani **Athira, S.**

Date: 12/10/2018 (2016-11-128)

CERTIFICATE

Certified that this thesis entitled **"MANAGEMENT OF BACTERIAL WILT DISEASE OF TOMATO BY THE ROOT ENDOPHYTIC FUNGUS *Piriformospora indica*, RHIZOBACTERIA AND BACTERIAL ENDOPHYTES"** is a record of research work done independently by Ms. Athira, S. (2016-11-128) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

Vellayani

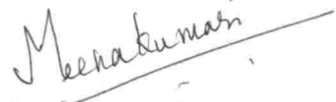
Date: 12/10/2018

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

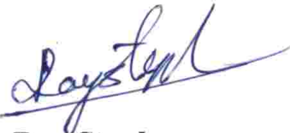
CERTIFICATE

We, the undersigned members of the advisory committee of Ms. Athira, S. (2016-11-128) a candidate for the degree of **Master of Science in Agriculture** with major in Agricultural Microbiology, agree that the thesis entitled "**MANAGEMENT OF BACTERIAL WILT DISEASE OF TOMATO BY THE ROOT ENDOPHYTIC FUNGUS *Piriformospora indica*, RHIZOBACTERIA AND BACTERIAL ENDOPHYTES**" may be submitted by Ms. Athira, S. (2016-11-128), in partial fulfillment of the requirement for the degree.

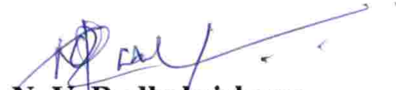
Dr. K. N. Anith
(Chairman, Advisory Committee)
Professor (Microbiology)
Department of Agricultural Microbiology
College of Agriculture, Vellayani



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



Dr. Roy Stephen
(Member, Advisory Committee)
Professor (Plant Physiology)
Department of Plant Physiology
College of Agriculture, Vellayani



Dr. N. V. Radhakrishnan
(Member, Advisory Committee)
Professor (Plant Pathology) and Head
Coconut Research Station
Balaramapuram, Thiruvananthapuram

(EXTERNAL EXAMINER)
Dr. Veena, S. S.
Principal Scientist
ICAR- CTCRI
Sreekaryam,
Thiruvananthapuram

Acknowledgement

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

At the very outset, I would gratefully bow my head before the great demiurge, who have guided me through all these scuffles and hurdles towards this marvelous endeavour.

With cordial gratitude I thank Dr. K. N. Anith, Chairman of the research and my mentor whose sedulous support, enthusiastic motivation and unprecedented erudition succored me in realizing this venture. His timely advice and enlightenment inspired me a lot in vanquishing the hurdles I encountered during the course of the study as well as in writing this thesis.

I extend my deepest indebtedness to Dr. K. S. Meenakumari, Professor and Head, Department of Agricultural microbiology for her unceasing support, valuable advice and expert guidance throughout the course of the study.

I convey my sincere thankfulness to Dr. N. V. Radhakrishnan, Professor and Head, Coconut Research Station, Balaramapuram, whose proficiency in the subject and whole hearted encouragement have helped me a lot in accomplishing this venture.

I express my earnest gratefulness to Dr. Roy Stephen, Professor, Department of Plant physiology for inspiring me throughout the course with his affirmative and charming disposition.

I express my ineffable thanks to my beloved Ammu for always being there for me throughout all pleasures and struggles of this two year Journey.

I would not have completed this work without the whole hearted love, support and assistance offered by Aswini chechi.

I would like to express my wholehearted gratefulness towards my favourite friends Nisha, Pathu, Jacob, Vishnu, Christy for their endless support, love and company all along which made my journey memorable.

It was a great experience to share this venture along with my batchmate, GKG whose immense support and cheerful company helped me stay positive throughout the course work.

I am obliged to my seniors Nadiya chechi and Nyshanth chettan for their advices and support. I also wish to recall the support offered by my dear juniors Jithu, Divya, Nandana, Riyas, Shubham and Abhijith for their unvarying support and co-operation.

This expedition would have been incomplete without the vehement encouragement by the microbiology team members Ajith, Subha chechi, Viji chechi, Bindu chechi, Santhi chechi, Vini, Jasmi, Bindhu aunty, Anju mam and Santhosh chettan.

Finally, I would like to express my deepest and immense gratitude to my dearest Achan, Amma, Monu, Nikki, Keethu, Anji, Revu, Kavu and Anjali for staying with me diligently and patiently through all those ups and downs I faced during the study.

Athira

CONTENTS

Sl. No.	CHAPTER	Page No.
1.	INTRODUCTION	1
2.	REVIEW OF LITERATURE	5
3.	MATERIALS AND METHODS	28
4.	RESULTS	43
5.	DISCUSSION	53
6.	SUMMARY	69
7.	REFERENCES	73
	APPENDICES	
	ABSTARCT	

7

LIST OF TABLES

Table No.	Title	In between pages
1	Bioagents used in the present study	31-32
2	Population of *Ralstonia solanacearum* in CPG broth and OD value at 660 nm measured at 2 h interval.	44-45
3	Population of *Ralstonia solanacearum* in the serially diluted samples from overnight grown CPG broth and OD value at 660 nm.	44-45
4	Interaction of bacterial bioagents and *Ralstonia solanacearum* assessed by cross streak plate assay	44-45
5	Interaction of bacterial bioagents and *Ralstonia solanacearum* assessed by agar plug diffusion technique	45-46
6	Interaction of bacterial bioagents and *Ralstonia solanacearum* assessed by disc diffusion methods	45-46
7	Interaction of bacterial bioagents and *Ralstonia solanacearum* assessed by spot on lawn method	45-46
8	Effect of the culture filtrate of bioagents on *Ralstonia solanacearum* assessed by agar well assay	46-47
9	Effect of the culture filtrate of bioagents on *Ralstonia solanacearum* assessed by disc diffusion method	46-47
10	*In vitro* assessment for compatability of bacterial bioagents with *Piriformospora indica* by dual culture plate assay	46-47
11	Population assessment of bacterial bioagents during co-culturing with *Piriformospora indica*	47-48
12	Incidence of bacterial wilt in the variety Naveen observed 7 days after challenge inoculation	48-49
13	Incidence of bacterial wilt in the variety Naveen observed 14 days after challenge inoculation	48-49

14	Incidence of bacterial wilt in the variety Naveen observed 21 days after challenge inoculation	48-49
15	Incidence of bacterial wilt in the variety Naveen observed 7 days after challenge inoculation	48-49
16	Incidence of bacterial wilt in the variety Naveen observed 14 days after challenge inoculation	48-49
17	Incidence of bacterial wilt in the variety Naveen observed 21 days after challenge inoculation	49-50
18	Root colonization by *Piriformospora indica* in the tomato variety Naveen	49-50
19	Incidence of bacterial wilt in the variety Vellayani Vijay observed 7 days after challenge inoculation	50-51
20	Incidence of bacterial wilt in the variety Vellayani Vijay observed 14 days after challenge inoculation	50-51
21	Incidence of bacterial wilt in the variety Vellayani Vijay observed 21 days after challenge inoculation	50-51
22	Incidence of bacterial wilt in the variety Vellayani Vijay observed 7 days after challenge inoculation	50-51
23	Incidence of bacterial wilt in the variety Vellayani Vijay observed 14 days after challenge inoculation	50-51
24	Incidence of bacterial wilt in the variety Vellayani Vijay observed 21 days after challenge inoculation	51-52
25	Biometric observation of tomato variety Vellayani Vijay treated with various bioagents and their combinations	51-52
26	Comparison of *in vitro* antagonism by bioagents against *Ralstonia solanacearum*	56-57
27	Comparison of bacterial wilt incidence (%) in the tomato varieties Naveen and Vellayani Vijay on inoculation with bioagents individually	60-63
28	Comparison of bacterial wilt incidence (%) in the tomato varieties Naveen and Vellayani Vijay on inoculation with bioagents in combination with *Piriformospora indica*	65-66

LIST OF FIGURES

Figure No.	Title	In between pages :
1	Relationship between optical density and population of *R. solanacearum* in batch culture system	44-45
2	Relationship between the optical density and population of *R. solanacearum* in alternate assay	44-45
3	Disease progression in the variety Naveen treated with individual bioagents after challenge inoculation	62-63
4	Disease progression in the variety Naveen treated with combination of bioagents after challenge inoculation	62-63
5	Disease progression in the variety Vellayani Vijay treated with individual bioagents after challenge inoculation	62-63
6	Disease progression in the variety Vellayani Vijay treated with combination of bioagents after challenge inoculation	62-63
7	Comparison of disease incidence in the tomato varieties Naveen and Vellayani Vijay treated with individual bioagents after 7th day of challenge inoculation.	63-64
8	Comparison of disease incidence in the tomato varieties Naveen and Vellayani Vijay treated with individual bioagents after 14th day of challenge inoculation	63-64
9	Comparison of disease incidence in the tomato varieties Naveen and Vellayani Vijay treated with individual bioagents after 21st day of challenge inoculation	63-64
10	Comparison of disease incidence in the tomato varieties Naveen and Vellayani Vijay treated with combination of bioagents after 7th day of challenge inoculation	65-66

11	Comparison of disease incidence in the tomato varieties Naveen and Vellayani Vijay treated with combination of bioagents after 14th day of inoculation	65-66
12	Comparison of disease incidence in the tomato varieties Naveen and Vellayani Vijay treated with combination of bioagents after 21st day of challenge inoculation	65-66
13	Percentage disease suppression over pathogen inoculated control in the tomato varieties treated with individual bioagents on 21st day after challenge inoculation	66-67
14	Percentage disease suppression over pathogen inoculated control in the tomato varieties treated with combination of bioagets on 21st day after challenge inoculation	66-67
15	Plant height of the tomato variety Vellayani Vijay on inoculation with the bioagents	67-68
16	Leaf number of the tomato variety Vellayani Vijay on inoculation with the bioagents	67-68
17	Shoot fresh weight of the tomato variety Vellayani Vijay on inoculation with the bioagents	67-68
18	Shoot dry weight of the tomato variety Vellayani Vijay on inoculation with the bioagents	67-68
19	Root fresh weight of the tomato variety Vellayani Vijay on inoculation with the bioagents	67-68
20	Root dry weight of the tomato variety Vellayani Vijay on inoculation with the bioagents	67-68

LIST OF PLATES

Plate No.	Title	In between pages:
1.	Nursery production of tomato plants in pro-tray cavities	36-37
2.	General view of the experimental plot	38-39
3.	Tomato plants with typical bacterial wilt symptoms in the field	43-44
4.	Bacterial ooze from the stem portions of infected plants	43-44
5.	Colonies of *Ralstonia solanacearum* on SMSA agar medium	43-44
6.	Wilt symptom in tomato plant artificially inoculated with virulent strain of *Ralstonia solanacearum*	44-45
7.	Cross streak plate assay for checking antagonism between bacterial bioagents and *Ralstonia solanacearum*	44-45
8	Direct methods for checking antagonism between bacterial bioagents and *Ralstonia solanacearum* A- Agar plug diffusion method; B- Disc diffusion method; C – Spot on lawn method	45-46
9	Indirect methods used for checking antagonism of bacterial bioagents and the fungal endophyte against *Ralstonia solanacearum* A. Agar well diffusion method; B. Disc diffusion method	46-47
10	*In vitro* assessment of compatibility between bacterial bioagents and *Piriformospora indica* by dual culture plate assay	46-47
11	Colonization of *Piriformospora indica* within the root cortex cells of tomato palnts	49-50
12	Assessment of plant growth promotion by bioagents in the tomato variety Vellayani Vijay	51-52
13	Assessment of plant growth promotion by bioagents in the tomato variety Vellayani Vijay	51-52

LIST OF APPENDICES

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

LIST OF ABBREVIATIONS AND SYMBOLS USED

et al.	And other co-workers
CPG	Casamino acid-Peptone-Glucose medium
cm	Centimetre
cfu	Colony forming units
CRD	Completely Randomised Design
DAI	Days after inoculation
DAP	Days after planting
^{0}C	Degree Celsius
Fig.	Figure
g	Gram
h	Hours
KB	King's B
μl	Micro litre
μg	Microgram
ml	Milli litre
mm	Milli metre
mg	Milligram
min	Minutes
viz.	Namely
No.	Number
NA	Nutrient Agar
%	Percent
PDA	Potato Dextrose Agar
SMSA	Semiselective Medium from South Africa
Sl.	Serial
sp or spp.	Species (Singular and Plural)
SDW	Sterile Distilled Water
var.	Variety

Introduction

1. INTRODUCTION

Tomato is one among the most important horticultural crops around the world which is best fit to grow in warm and dry environment. Besides its nutritional value, presence of a predominant level of antioxidants including vitamin C, β carotene and lycopene in tomato help in defense against oxidative stress and enhance disease prevention. It is also considered as one of the model crops for studies on biotic and abiotic stress as well as agronomic research.

One of the major biotic constraints affecting the commercial production of tomato is bacterial wilt disease caused by *Ralstonia solanacearum.* The pathogen is present all over the world with a wide host range (Norman *et al.*, 2009). Bacterial wilt is a soil borne disease that causes complete crop loss when there is a severe outbreak. Even though integrated management including cultural practices, crop rotation and use of resistant cultivars provide limited success in combating the disease, bacterial wilt still threatens commercial tomato production in India and worldwide (Vanitha *et al.*, 2009). Though there are many resistant varieties, most of the popular cultivated varieties having high yield are susceptible to the pathogen. Many of the resistant cultivars are less preferred by farmers as they are low yielders and have location specificity.

Conventional management practices using chemical agents and use of cultural practices have very limited application in the management of bacterial wilt disease in tomato. There is at present, no effective management strategy for the disease. Chemical treatments used for the management of bacterial wilt disease are expensive compared to the conventional fungicides and insecticides used against fungal pathogens and insect pests. Moreover, soil fumigation with certain chemicals that reduced the bacterial wilt incidence to some extent, are now not permitted due to stringent environmental restrictions (Mao *et al.*, 2014).

Many of the tropical vegetable crops such as tomato, brinjal, chillies etc. are transplanted into the main field after raising the seedlings either in poly bags or transplant trays (pro-trays). It has been found that this process ensures uniform crop stand as well as early flowering in the field. Application of biological control agents and microbial inoculants during the pro-tray production of vegetable crops assures effective colonization and survival of these bio agents in the developing root system.

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) (Kloepper *et al.*, 1980). PGPR have a positive influence on plant growth especially under stress conditions. They exert beneficial effects on plant development via direct or indirect mechanisms (Whipps, 2001). They directly enhance plant growth by a variety of mechanisms such as production of siderophores for iron uptake, solubilization of minerals such as phosphorus and synthesis of phytohormones. Indirectly they enhance plant growth and health by suppressing phytopathogens, utilizing mechanisms such as antagonism, competition for space and nutrients and induction of systemic resistance (ISR) in host plant against a broad spectrum of plant pathogens (Hyakumachi *et al.*, 2013).

Several reports suggest that these root colonizing bio-agents also colonize the internal tissues of the plant roots thus making them to be considered as endophytes. Endophytes are those microorganisms that reside within growing plant tissues without doing substantive harm or gaining benefit other than residency. They include bacteria and fungi that can be isolated from surface-disinfected plant tissues or extracted from inside the plant which does not cause any visible harm (Hallmann *et al.*, 1997). Endophytes are considered to be important candidates for developing biocontrol agents against plant diseases as they are highly adapted to the host plant

system and thus effectively deter the attack of the invading pathogens (Johri, 2006). Colonization by endophytes, both fungi and bacteria, are reported to enhance the disease resistance of many crop plants (Achatz *et al*., 2010; Meena *et al*., 2010). There are reports about the occurrence of endophytic colonization by biological control bacteria including the well known *Pseudomonas fluorescens* in many crops.

Arbuscular Mycorrhizal Fungi (AMF) possesses disease suppression with regard to soil borne diseases. Substantial growth promotion in many crops was also observed upon inoculation with AMF. *Piriformospora indica* Varma, Verma, Kost, Rexer and Franken sp. nov. is an axenically cultivable plant growth promoting endosymbiont, which mimics the capabilities of AMF (Verma *et al*., 1998). *P. indica* is reported to have 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* and several other economically important crops (Varma *et al*., 2012). Many plant species respond positively to inoculation with this fungus and hence, the fungus has multiple biotechnological applications. For example, *P. indica* has been used as a biological hardening agent for tissue cultured plantlets (Anith *et al*., 2011). This root endophyte improves the disease resistance against many plant pathogens also. The endophytic fungus also improves the drought tolerance in many plants (Deshmukh and Kogel, 2007; Serfling *et al*., 2007; Sherameti *et al*., 2008)

Use of biological agents as a consortium or mixture is more advantageous than using their components individually (Schisler *et al*., 1997). However, the *in vitro* and *in vivo* interactions of these bio agents need to be studied for efficient use. Developing a biological control and plant growth promoting system by formulating a root endophytic microbial consortium having efficient endophytic colonizers which could be used for treating seedlings in the transplants will be of great practical significance. Use of biocontrol agents against many crop pests and diseases are in

practice. Exploiting microbial strains with multiple functional capabilities is an attractive area of research in this context. The important criteria for selecting a bacterial biocontrol agent against soil borne diseases include ability of the agent to effectively colonize the target crop plant, inhibition of rhizosphere pathogens and thereby decreasing disease incidence. At the same time, they may also positively influence the crop growth and health.

Biological control is an emerging management strategy, and evolving a consortium of bioagents with multiple traits is an important aspect in its future development. Developing an endophyte consortium consisting of bacteria and fungi for biotic stress management as well as plant growth promotion by their effective application over space and time during crop production, starting from the nursery will be a practical approach for maintaining plant health. The present study was undertaken with the above key points in mind with the main objective to assess the potential of the root endophytic fungus *Piriformospora indica,* plant growth-promoting rhizobacteria and bacterial endophytes in suppressing bacterial wilt incidence in tomato.

Review of Literature

2. REVIEW OF LITERATURE

Tomato (*Solanum lycopersicum* L.), the most widely cultivated vegetable crop in the world next to potato, is universally accepted as a "protective food". Due to the abundance of vitamins A and C, minerals, carotenoids and the antioxidant lycopene, tomato plays a major role in human diet (Sangrit *et al.*, 2011). India ranks second in tomato production next to China (NHB, 2016). Tomato is grown in 8.09 lakh ha with a production of 196.97 lakh MT and productivity of 24.4 MT ha^{-1} in 2014-2015 (NHB, 2017).

Bacterial wilt disease is one of the major constraints in the commercial production of tomatoes worldwide. *Ralstonia solanacearum*, the causal agent is a Gram negative soil-borne bacterium which causes pernicious wilt disease with a wide expanding host range of hundreds of susceptible species around the world (Kelman *et al.*, 1994). Vast geographic distribution, abnormally extended host range and aggressiveness make bacterial wilt a highly destructive plant disease (Prior *et al.*, 1998).

Host range of *R. solanacearum* includes economically important crops like potato, tomato, tobacco, banana and peanut (Hayward, 1991). The pathogen causes remarkable level of yield loss and monetary loss (Poussier *et al.*, 1999).

The bacterium generally invade the host plant through wounds or natural openings, gradually penetrating the xylem vessels, colonizing the stem and spreading further and producing wilt symptoms (Kelman, 1953). The disease symptom is characterized by flaccidity of the younger foliage of infected plants during the hottest hour of the day. Under conducive environment, the whole plant wilts whereas in less favorable environment, the disease proceed gradually beginning with stunting followed by appearance of adventitious roots on the collar region. The affected plants further develop vascular discoloration with bacterial ooze when dissected (Gota,

1992). Vasse *et al.* (1995) observed that the pathogen transcended a very high cell density of 10^9 CFU g^{-1} in infected host tissues.

R. solanacearum persist in soil, with or without susceptible hosts (Kelman, 1953; Buddenhagen and Kelman, 1964). Dukes *et al.* (1965) suggested the possibility of heavy losses in tomato planted on cleared land due to the long-term survival of the pathogen in soil even without the presence of known hosts. Devi *et al.* (1981) reported the persistence of the pathogen in the rhizosphere of weeds. The bacterium was seen widening its host range by infecting non-host plants (Granada and Sequeira, 1983). After colonization, bacterial cells are shed from roots creating a pathway for the pathogen to re-enter the soil (Heyward, 1991).

According to the ability of *R. solanacearum* strains to infect different host plants, they are classified into different racial pattern. Race 1 includes strains of wide host range including solanaceous plants and weed hosts, race 2 is confined to *Heliconia* and triploid banana, race 3 is restricted to potato, race 4 to ginger and race 5 confined to mulberry (He *et al.*, 1983). Based on the ability of the strain to metabolize or oxidize specific hexose sugars and disaccharides, the pathogen has been divided into five groups (Hayward, 1991). *R. solanacearum* strains were further phylogenetically analyzed based on molecular pattern and sequence data generated by 16S-23S internal transcribed spacer (ITS) and are distributed into four major groups. The pathogen is further classified into sequevars based on endoglucanase variation in the sequence (Fegan and Prior, 2005).

2.1. MANAGEMENT OF BACTERIAL WILT

Conventional approaches for the management of bacterial wilt disease based on physical, chemical and cultural practices have been found to be inefficient in producing expected results. The viability, adaptability and genetic diversity of *R. solanacearum* render practical difficulty in the effective management of the

disease (Elphinstone, 2005). The use of resistant cultivars has met with limited success due to the emergence of newly infective *R. solanacearum* strains (Chave *et al.*, 2017).

2.1.1. Cultural Practices

2.1.1.1. *Use of Resistant Varieties*

Bacterial wilt resistant varieties of economically important crops were produced by breeding techniques considering different factors such as availability and diversity of resistance sources, variability of pathogenic strains, plant pathogen interactions, breeding and selection methodology (Boshou, 2005; Elphinstone, 2005).

Resistant varieties exhibits high tolerance by not producing any visible symptom even when severely infected with the bacterial wilt pathogen (Prior *et al.*, 1996). In resistant tomato varieties, the migration of pathogen from protoxylem to other xylem tissues was found to be restricted which in turn suppressed bacterial multiplication inside the stem (Nakaho *et al.*, 2004). The introduction of *Arabidopsis npr*1 gene, the key gene responsible for the transduction of SA and JA/ethylene mediated signals that triggers systemic acquired resistance into tomato cultivar H7996 amplified bacterial wilt resistance of the host by 70 per cent within 28 days of inoculation (Lin *et al.*, 2004).

Bacterial wilt resistance seem to have a negative correlation with yield and quality of the produce and newer biotechnological approaches like genetic transformation are yet to be exploited in this area to produce resistant varieties with better yield (Yuliar *et al.*, 2015).

2.1.1.2. ***Soil Amendments***

Manipulation of soil conditions by amendment with various organic and inorganic substrates tends to influence the survival and population build up of many soil borne plant pathogens. Attempts have been made to utilize this principle for the management of bacterial wilt disease in several locations.

Application of calcium into soil as fertilizer at higher doses tends to curtail the severity of wilt incidence and reduce the population of the pathogen in the host stem (Yamasaki and Hoshina, 1995; Yamasaki *et al.*, 2000). The combined application of nitrogen, phosphorus and potassium reduced bacterial wilt to the extent of 29 percent whereas the combination of nitrogen and potassium reduced the disease by 50 percent (Lemaga *et al.*, 2005). Combined application of rock dust with organic fertilizers tends to show diminishing rate of wilt incidence in tomato (Li and Dong, 2013). Silicon and chitosan when applied together initiated boosting of resistance in tomato against bacterial wilt (Kiirika *et al,* 2013).

2.1.1.3. ***Crop Rotation***

Even though crop rotation with non susceptible host provides control of the pathogen to some extent, its wide host range is an obstacle in this approach (Kelman, 1953). Susceptible tomato varieties when cultivated in succession with corn, okra, cowpea and resistant tomato hinder the commencement of wilt disease by one to three weeks with a decrement of 20-26 percent disease extremity (Adhikari and Basnyat, 1998). The root exudates of Chinese chive help in avoiding the pathogen from invading tomato plants when cultivated alongside each other (Yu, 1999).

2.1.2. Physical Methods

Physical methods in controlling *R. solanacearum* encompass mainly soil solarization, hot water treatment, heat treatment, cold treatment, biofumigation and application of high voltage electrostatic field.

Akiew and Trevorrow (1994) delineated that tobacco plants cultivated in winter season show lesser disease development than those planted in spring season suggesting the influence of climate on wilt incidence.

Anith *et al.* (2000) reported that irrigation followed by soil solarization at 45 days prior to planting as an effective strategy in reducing bacterial wilt disease incidence in ginger in a wilt sick field of Kerala. Significant reduction in soil population of the pathogen was observed during the period of solarization. Incidence of bacterial wilt was found to be reduced effectively in tomato when soil was mulched with plastic 60 days before planting (Vinh *et al.*, 2005). Kumar and Sood (2001) reported that rhizome solarization of ginger can reduce the bacterial wilt incidence up to 90-100 per cent. In tomato field, a decline in the population of wilt pathogen was observed after soil solarization in a study conducted by Baptista *et al.* (2006, 2007). Bacterial population in infected soil was reduced by heat treatment at 45°C and 60°C for 2 days and 2 h respectively inhibited 97 per cent of infection and the disease incidence up to 75 per cent in tomato (Kongkiattikajorn and Thepa, 2007).

Suppression of bacterial wilt by physical method was thought to be effective due to killing of the pathogens by high or low temperatures (Scherf *et al.*, 2010).

Biofumigation or Biological Soil Disinfection (BSD) which makes use of volatile compounds like organic acids or heavy metal ions released from plant residues for the suppression of soil-borne fungal pathogens was studied by Kirkegaard *et al.* (1996). This has been found to be moderately effective against the bacterial wilt pathogen.

Potting mixture infected with *R. solanacearum* when treated with volatile essential oils such as thymol, palmarosa oil and lemon grass oil reduced the population of the pathogen to undetectable levels. No incidence of bacterial wilt was observed in tomato plants transported to soil amended with thymol, palmarosa and lemon grass oil, though the presence of the pathogen was detected in the plants (Pradhanang *et al.*, 2003). Study conducted by Ji *et al.* (2005) revealed a significant reduction in the number of wilted plants when the planting medium was treated with thymol and palmarosa oil. The percentage wilt incidence has been reported as 33.1 per cent for thymol and 48.1 per cent for palmarosa treated pots.

The composite application of thymol and Acibenzolar-S-Methyl (ASM) in tomato was found to be more effective in reducing the disease incidence when compared to their solitary application (Hong *et al.*, 2011).

R. solanacearum when exposed separately to High Voltage Electrostatic Field (HVEF) and Radio Frequency Electromagnetic Field (RFEF) for more than 40 minutes, the total number of bacteria was decreased by 84 per cent and 95 per cent respectively (Wu *et al.*, 2007).

2.1.3. Chemical Methods

Though chemical control methods were accepted widely due to its greater net benefit compared to other methods, the unreasonable use of agrochemicals by the farmers is thought to be a looming threat on environmental health (Edwards-Jones, 2008). Chemical control of bacterial wilt is usually accomplished by using pesticides and plant activators.

A combination comprising of methyl bromide, 1, 3-dichloropropene and chloropicrin was found to reduce wilt incidence in tobacco by 28 percent (Fortnum and Martin, 1998). Suppression of bacterial wilt disease was observed in tomato plants treated with pesticides, fumigants and plant activators that induced systemic

resistance and thereby increasing the yield by 1.7 to 2.5 folds than that of control (Santos *et al.*, 2006). Norman *et al.* (2006) proved the bacteriostatic action of phosphoric acid solution against bacterial wilt pathogen.

Use of chemicals like bleaching powder (Sharma and Kumar, 2000), bactericides (Khanum *et al.*, 2005), weak acidic electrolyzed water (Yamasaki *et al.*, 2006) and antibiotics (Lin *et al.*, 2010) for decreasing microbial load in soil was found to be effective in suppressing bacterial wilt disease.

Seedling vigor and disease tolerance in tomato against *R. solanacearum* was boosted by seed treatment with dilute sodium chloride solution (Nakaune *et al.*, 2012).

In vitro bactericidal activities of six chemical pesticides were tested against *R. solanacearum* by Lee *et al.* (2012). Though all of them showed *in vitro* inhibitory action, only four namely copper hydroxide, copper hydroxide-oxadixyl mixture, copper oxychloride-kasugamycin mixture and streptomycin-validamycin were effective in suppressing the disease under pot culture experiments. It was also suggested that copper compounds, antibiotics and essential oil possess ability to control tomato wilt.

A mixture of peroxyacetic acid, hydrogen peroxide and acetic acid was evaluated *in vitro*, against *R. pseudosolanacearum*. It was also found that 0.01 per cent of the mixture when applied on to the detached leaves of two cultivars delayed the wilting symptom significantly when challenge inoculated with *R. pseudosolanacearum*. Similarly, soil-drenching of 1 per cent of the above mixture into the pots significantly reduced wilt incidence in tomato seedlings (Hong *et al.*, 2018).

2.1.4. Biological Methods

Biological control encompasses the use of biological organisms, especially microorganisms including bacteria and fungi in disease or pest management (Yuliar *et al.*, 2015). Biocontrol involving microbial agents are thought to be superior to other control methods due to their following properties: (a) self-sustaining potential (b) ability to multiply on its own after initial establishment (c) decreased use of non-renewable resources (d) prolonged and eco-friendly disease suppression (Quimby *et al.*, 2002; Whipps and Gerhardson, 2007). At present, the potential of biological control agents like rhizobacteria, endophytic bacteria and fungi in controlling wilt disease have been exploited only to an extent of about 50 per cent (Yuliar *et al.*, 2015).

Biocontrol agents used against bacterial wilt include avirulant mutants of *R. solanacearum* (McLaughlin and Sequeira, 1988; Trigalet and Trigalet, 1990) antagonistic rhizobacteria (Ciampi-Panno *et al.*, 1989) and endophytic bacteria (Tan *et al.*, 2013).

2.1.4.1. *Rhizobacteria as Biocontrol Agents*

Some strains of *Pseudomonas fluorescens* was found to actively suppress the occurrence of tomato bacterial wilt when introduced to plant rhizosphere by root dipping (Aino *et al.*, 1993).

Ginger seed treatment with *P. fluorescens* strain EM85 together with soil solarization was able to suppress the incidence of bacterial wilt compared to the untreated control (Anith *et al.*, 2000). As reported by Kumar and Sood (2001), the antagonistic rhizobacterial strains of *P. fluorescens* and *Bacillus cereus* when incorporated into the soil before soil solarization, reduced the population of *R. solanacearum*.

Bacillus pumilus SE 34 and *Pseudomonas putida* 89B6 significantly reduced bacterial wilt incidence when applied to the transplants at the time of seeding and one week prior to inoculation with *Ralstonia solanacearum* (Anith *et al.*, 2004). In tomato cultivated under greenhouse conditions, three strains of PGPR *viz. Serratia* sp J2, *Pseudomonas fluorescens* J3 and *Bacillus* sp BB11 were evaluated for antagonism against *R. solanacearum* and were found to be effective in controlling the disease and increasing the yield (Guo *et al.*, 2004). *Bacillus vallismortis* EXTN-1 controlled the bacterial wilt incidence in tomato by impeding the movement of the pathogen within the stem of the bacterized plants (Park *et al.*, 2007).

In vitro evaluation of 118 rhizobacterial strains from Ethiopia was done for antagonistic action against *R. solanacearum* and six promising strains were further tested *in planta* for bacterial wilt suppression by Lemessa and Zeller (2007). Tomato plants when inoculated with *Pseudomonas* sp APF1 and *Bacillus subtilis* B2G strains showed a reduction in area under disease progress curves by 60 per cent and 56 per cent respectively. As reported by Messiha *et al.* (2007) there was a decreased disease suppression in potato against bacterial wilt by application of *Stenotrophomonas maltophilia.* Bacterial wilt suppression was achieved by application of *Bacillus*-based biocontrol agents on mulberry (Ji *et al.*, 2008) and tobacco (Maketon *et al.*, 2008).

In *Solanum tuberosum*, out of 120 strains of PGPR screened, six were found to be antagonistic against *R. solanacearum.* Three rhizobacterial strains *viz.*, PFMRI (B*acillus subtilis*), BS-DFS and PF9 (*Pseudomonas macerans*) effectively reduced wilt incidence by 82.7, 66.2, and 65.7 per cent respectively and area under disease progress curve by 78.6, 66, and 64.3 per cent respectively (Aliye *et al.*, 2008). The biocontrol agent *Pythium oligandrum* was found to be suppressive against bacterial wilt caused by *R. solanacearum* in tomato (Masunaka *et al.*, 2009).

Bacillus megaterium and *Enterobacter cloacae* isolated from soil, enhanced the bacterial wilt disease suppression in chilli with an increase in plant growth (Nguyen and Ranamukhaarachchi, 2010).

Pseudomonas fluorescens, *P. putida* and *Bacillus subtilis* isolated from root zone of tomato increased the seed germination up to 15 per cent over untreated control when tested *in vitro* and *in vivo* against the most virulent strain of *R. solanacearum* isolated from naturally wilted plants (Seleim *et al.*, 2011). *Pseudomonas mallei* RB64 tend to reduce 65 per cent of bacterial wilt incidence and increase the yield up to 75 per cent in eggplant (Ramesh and Phadke, 2012).

Pseudomonas fluorescens act as a bio control agent by reducing the percentage of wilt incidence in eggplant. Population densities of the pathogen and antagonist showed negative correlation with each other along with negative correlation between percentage wilt incidence and population density of the antagonist (Chakravarty and Kalita, 2012).

About 60 isolates of *Bacillus subtilis* collected from different locations of China were screened for antagonism against *R. solanacearum* and of these, six isolates exhibited above 50 percent efficacy against bacterial wilt pathogen on tomato when tested in green house condition (Chen *et al.*, 2013).

Study conducted by Hyakumachi *et al.* (2013) detailed the bio control potential of *Bacillus thuringiensis* which significantly reduced bacterial wilt incidence in tomato plants to less than one third when compared to control. The same result was reproduced when pretreated with cell-free extract of *B. thuringiensis* along with the reduction in population densities of *R. solanacearum* in the stem tissues.

Kurabachew and Wydra (2013) conducted an experiment in which 150 isolates of rhizobacteria were tested against *R. solanacearum* induced wilt in tomato. Thirteen isolates of *Pseudomonas* spp., *Serratia marcescens* and *Bacillus cereus* were found to

be effective in controlling the disease. *Bacillus cereus* and *Pseudomonas putida* reduced bacterial wilt to an extent of 46.8 per cent and 44.7 per cent respectively. In a related experiment, inoculation of tomato with the rhizobacterial strain *Bacillus pumilis*, lowered the bacterial wilt incidence by 26.7 per cent in moderately resistant variety and by 22.2 per cent in susceptible variety (Kurabachew and Wydra, 2014).

In a study conducted by Maji and Chakrabartty (2014), mixed inoculation of antagonist *Pseudomonas* sp BH25 with the wilt pathogen *R. solanacearum* on to tomato seeds showed 75 per cent seedling emergence as compared to that of pathogen inoculated control (40 per cent) and absolute control (76 per cent).

Almoneafy *et al.* (2014) reported the production of IAA, siderophores and biofilm by rhizobacterial isolates, *Bacillus amyloliquefaciens* D29, *B. amyloliquefaciens* Am1, *B. subtilis* D16 and *B. methylotrophicus* H8. Culture filtrate of these strains effectively suppressed the growth and bio film formation of *R. solanacearum in vitro* and exhibited antibacterial activity against the pathogen besides its ability to stimulate tomato growth *in vivo*.

Greenhouse experiments conducted in potato showed that use of *Pseudomonas fluorescens* Pf11, *Paenibacillus* sp Pb28 and *Pseudomonas putida* Pp17 helped in reducing wilt disease incidence by 38.58, 55.56 and 51.50 per cent respectively (Kheirandish and Harighi, 2015).

Studies by Wu *et al.* (2017) found that organic acids helped in increasing the population and pathogenicity of *R. solanacearum* and also favoured the biocontrol rhizobacerium *Bacillus amyloliquefaciens* SQYUV 162. Co-inoculation of pathogen and the bio control bacteria in the presence of organic acids preferably favoured the growth of the latter. This in turn reduced the disease incidence by depleting the available nutrients and competing with the pathogen. The authors suggested the

importance of root exudates in the process of biological control by rhizobacterial strains.

Biocontrol bacterial isolates TCR112 (*Ralstonia* sp) and TWR114 (*Mitsuaria* sp) when used for drenching the soil at weekly intervals in tomato, reduced disease incidence by 57.2 and 85.8 per cent respectively in first year and 57.2 and 35.3 per cent respectively in second year (Marian *et al.*, 2018). Both the bacterial isolates had stable colonization capacities at rhizosphere as well as within the plants. This has resulted in the effective reduction of wilt pathogen population in the rhizosphere as well as in the crown region of the plants.

Antagonistic effects of rhizosphere-associated *Bacillus velezensis* isolates (Y6 and F7) were tested against *R. solanacearum* under both *in vitro* and greenhouse conditions. Both the isolates exhibited strong antagonism against *R. solanacearum in vitro*. The two isolates were also noticed to give a reduction of about 29% and 35% reduction when treated with Y6 and F7 respectively where the control plant showed 65% wilt incidence (Cao *et al.*, 2018).

Plant growth-promoting rhizobacterial strain *Bacillus pumilus* WP8 was found to effectively control the bacterial wilt pathogen by preventing its movement from the root of tomato plants to above ground parts (Shen *et al.*, 2018). The heat resistant secondary metabolites produced by the biocontrol bacterium inhibited the motility of *R. solanacearum* and prevented the spread of the pathogen inside plant parts.

2.1.4.2. *Endophytic Bacteria as Biocontrol Agents*

Endophytes isolated from eggplant, cucumber and groundnut *viz.*, *Burkholderia cepacia* EB9, *Enterobacter* sp EB44, *Pseudomonas* sp EB67, *Enterobacter cloacae* EB89, *Bacillus* sp EC4 and *Bacillus* sp EC13 when inoculated into eggplant reduced the incidence of bacterial wilt by 70 per cent compared to the untreated control (Ramesh *et al.*, 2009).

Bacillus amyloliquefaciens Bg-C3, a bacterial endophyte isolated from mangroves had the potential to control bacterial wilt in *Capsicum* produced by *R. solanacearum* in pot and field trials. Antimicrobial activity was proposed to be due to the presence of a protein, resistant to protease K and heat (Hu *et al.*, 2010).

In a study conducted in Indonesia by Nawangsih *et al.* (2011) 41 non-plant pathogenic endophytic bacterial strains were isolated from healthy tomato plants and tested for their ability to promote plant growth and suppress bacterial wilt disease. Six selected isolates based on antagonistic effect on *R. solanacearum* when tested under green house conditions revealed that two isolates, BC4 (*Staphylococcus epidermidis*) and BL10 (*Bacillus amyloliquefaciens*) were promising bio control agents.

Two strains of *Streptomyces virginiae*, Y30 and E36 isolated from tomato plants were suggested as potential antagonists against *R. solanacearum.* The capability of the endophytes to produce siderophores and ACC deaminase activity has been implicated in the biological control. In the green house condition, both the strains significantly protected tomato seedlings against wilt pathogen (Tan *et al.*, 2011).

Amaresan *et al.* (2012) tested antagonism of nine isolates from tomato and seven isolates from chilli against *R. solanacearum* and found that the inhibition zone in plate assay was greater for the isolates from tomato (*Bacillus pumilus* BETL13, *Proteus vulgaris* BETS13, *Corynebacterium minutissimum* BETL9, *Staphylococcus delphini* BETL10, *Bacillus amyloliquefaciens* BETR11, *Bacillus cereus* BETS15) when compared to that of chilli. The isolates were also assessed for the production of siderophore, indole acetic acid, extracellular enzymes and phosphate solubilisers which may contribute to their biocontrol potential. Three endophytic isolates with inhibitory property against *R. solanacearum* showed plant growth promotion in tomato during bioassay under greenhouse conditions.

Cultivation dependent and independent methods were used to understand the endophytic microbial community status in *R. solanacearum* resistant or susceptible tomato cultivars by Feng *et al.* (2013). Diversity and abundance of endophytic bacteria was found to be more in the cultivar Xiahong-1 (resistant) than in Baoshi-5 (susceptible). Antagonistic bacteria from the former were more efficient in suppressing the bacterial wilt incidence than those from the latter.

Root associated native bacterial endophytes from both resistant and susceptible tomato cultivars were isolated and screened against *R. solanacearum* by Upreti and Thomas (2015). The number of isolates with putative bio control potential was more in the resistant variety Arka Abha than in the susceptible variety Arka Vikas. Antagonistic endophytes isolated from the resistant cultivar included *Pseudomonas oleovorans, Pantoea ananatis* and *Enterobacter cloacae*. It was suggested that the presence of more number of antagonistic bacteria within the roots of resistant cultivar may have some influence on the resistance against wilt pathogen.

James and Mathew (2015) were successful in isolating endophytic microbes, including bacteria from several tomato cultivars from Kerala. *In vitro* tests showed that 12 out of 79 bacterial isolates had antagonistic effect against the wilt pathogen. Five isolates amongst them also suppressed the wilt incidence under pot culture conditions.

2.1.5. Integrated Disease Management

Combining different types of control strategies in an integrated manner would always pay improved results in disease management. Instances of such results have been reported in bacterial wilt as well.

Studies focusing on the combination of crop rotation with a resistant cultivar and organic matter with a non-pesticide chemical like formaldehyde or bleaching

powder resulted to have increased efficiency in controlling the wilt disease incidence (Adhikari and Basnyat, 1998, Lemaga *et al.*, 2005).

Application of the plant activator, ASM together with rhizobacteria and soil amendment with S-H mixture reduced bacterial wilt incidence in tomato (Anith *et al.*, 2004). Significant reduction in bacterial wilt incidence was observed when a combination of Actigard (ASM) and S-H mixture was applied.

Biocontrol agents when combined with substrates such as sucrose, lysine and anaerobically digested slurry promoted root colonization of biocontrol agents, thereby suppressing bacterial wilt in tomato (Nion, 2008; Nion and Toyota, 2008).

In tomato, the combined application of *Pseudomonas fluorescens* Pf2 with ASM reduced wilt disease incidence to a greater extent (Abo-Elyousr *et al.*, 2012). Resistant cultivars of tomato when treated with endophytic bacteria having no antibiosis against the pathogen, facilitated the lowering of bacterial wilt incidence (Barretti *et al.*, 2012).

2.2. *Piriformospora indica* - PLANT GROWTH PROMOTING ROOT ENDOPHYTIC FUNGUS

Piriformospora indica is a novel phyto-promotional, biotrophic, mutualistic root endophytic fungus, found colonizing 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). When compared to other biofertilizers, *P. indica* is very simple to grow in a bioreactor and can be formulated effectively (Singh *et al.*, 2003; Qiang *et al.*, 2011).Though it belongs to the newly formed family Sebacinaceae in the order Sebacinales, it bears a close resemblance with the Arbuscular Mycorrhizal Fungi (Weiss *et al.*, 2004; Qiang *et al.*, 2011). The fungus promotes plant growth, increase the resistance against biotic stress and intensify the tolerance against abiotic stress (Harman, 2011; Varma *et al.*, 2012). The growth of *P.*

indica could be performed axenically in conventional media such as PDA/PDB (Varma *et al.*, 2012).

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).

2.2.1. *Piriformospora indica* and Plant Growth Promotion

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).

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). *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).

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).

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).

Enhanced production of asiaticosides has been demonstrated in *Centella asciatica* on inoculation with *P. indica*. Up regulation of squaline synthase and β-amyrin synthase, the key enzymes in the synthesis of asiaticoside, in inoculated plants have also been reported (Satheesan *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.

2.2.2. *Piriformospora indica* and Disease Management

P. indica was found to have efficient disease control over the virulent root and seed pathogen *Gaeumannomyces graminis* (Varma *et al.*, 2001; Serfling *et al.*, 2007). Barley plants when colonized by *P. indica* appear to be highly tolerant to salt stress and extremely resistant to pathogens (Waller *et al.*, 2005). *P. indica* protects the host from pathogenic fungal attack other than being a growth promoter. The protective effect of *P. indica* on barley was assessed under semi-natural conditions. *P. indica* exerted a positive effect on disease suppression against the two major cereal pathogens *Alternaria alternata* and *Colletotrichum falcatum*.

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). In field condition, the effect of *P. indica* against *Blumeria graminis f.* sp. *tritici* was assessed and the results showed a reduction in the disease severity (Serfling *et al.*, 2007).

Among the plants treated with *P. indica* and without *P. indica* when artificially inoculated with the pathogenic fungus *Pseudocercosporella herpotrichoides,* a significant reduction in the disease was observed in inoculated plants (Serfling *et al.*, 2007).

Infection by *Fusarium verticillioides* root parasite of maize was reduced by the protective action of *P. indica* (Kumar *et al.*, 2009).

In tomato plants colonized by *Piriformospora indica*, the incidence of *Verticillium* wilt was reduced by 30 percent (Fakhro *et al.*, 2010). Wheat seedlings when treated with both *P. indica* and *Fusarium* isolates, showed similarity in root

biomass and seed emergence with uninoculated control and the seedlings were free from visible symptoms of fungal attack (Rabiey *et al.*, 2015).

Substantial decrease in leaf blight disease incidence in taro was reported by Lakshmipriya *et al.* (2016) on inoculating two cultivars, Sree Kiran and Muktakeshi with the root endophytic fungus *P. indica*. Increased activity of the defense enzymes *viz.*, chitinase, β-1,3 glucanase and total phenol has been observed in the inoculated plants compared to the non inoculated plants.

Varkey *et al.* (2018) reported that *P. indica* was able to suppress the incidence of root knot nematode infestation in tomato effectively than with two PGPR bacterial strains. However mixed inoculation of the endophytic fungus with the PGPR strains had less suppressive effects due to the antagonistic interaction between them.

2.3. PLANT GROWTH PROMOTING RHIZOBACTERIA AND TOMATO

Various symbiotic microorganisms like *Frankia* spp., *Rhizobium* spp. and asymbiotic microbes like *Bacillus*, *Azotobacter*, *Azospirillum* and *Klebsiella* are combined with the agricultural inputs to improve the crop growth as well as to increase the yield in a sustainable way (Staley and Drahos, 1994). Plant growth promoting rhizobacteria, a subdivision of total bacteria present around the root zone (rhizosphere) helps in attaining soil fertility and restoring soil health and thereby setting up a favorable environment for the plants to flourish (Lugtenberg and Kamilova, 2009). Rhizosphere bacteria were given considerable importance in agricultural production system after the concept of sustainable agriculture was put forth by Hayat *et al.* (2010).

The interactions existing between the rhizobacteria and associated plants are of three types (Whipps, 2001). A neutral interaction where there is no visible effect in the growth or physiology of the host (Beattie, 2006), negative interaction in which the plants are negatively influenced by the production of phytotoxic substance and a

positive interaction in which a positive effect created by the direct and indirect mechanism by the rhizobacteria (Bashan and de-Bashan, 2010).

PGPR helps in increasing the plant growth indirectly by altering microbial balance in the rhizosphere. Many of the PGPR strains have been implicated in reduction in population of plant pathogens and harmful rhizobacteria in the soil, with corresponding improvement in plant growth (Zehnder *et al.*, 1999).

PGPR are divided into two groups; extracellular plant growth promoting rhizobacteria (ePGPR) and intracellular plant growth promoting rhizobacteria (iPGPR) based on the degree of association with the plant roots (Martinez-Viveros *et al.*, 2010).

ePGPR and iPGPR get established in the root zone owing to their ability to adapt in wide range of environments, rapid multiplication and ability to metabolize an extended range of natural and xenobiotic compounds (Cook, 2002). The microorganisms in this category are again grouped into three groups that get involved in biological suppression of disease, enhancement in nutrient uptake and stimulating plant hormone production (Glick, 2003).

The PGPR strains isolated from tomato rhizosphere exhibited measurable antagonism to soil borne root pathogens, by producing indole acetic acid, cyanide, siderophores, phosphate solubilization and intrinsic resistance to antibiotics (De Brito *et al.*, 1995).

Tomato plants when treated with *Bacillus subtilis* and *Bacillus amyloliquefaciens* in combination with chitosan increased the height, fresh weight, fruit numbers and flower per plant (Murphy *et al.*, 2003).

A combination of *B. licheniformis* CECT 5106, *Pseudomonas fluorescens* CECT 5398 and *Chryseobacterium balustinum* CECT 5399 with LS213 (*Bacillus*

40

subtilis strain GB03, *B. amyloliquefaciens* strain IN937a and chitosan) increased the synergistic effect on growth promotion (Domenech *et al*., 2006).

Inoculation of the roots with PGPR isolate *Bacillus subtilis BEB-lSbs* (BS13) enhanced the yield per plant, texture, fruit weight and length and marketable quality in tomato (Mena-Violante and Olalde-Portugal, 2007).

Seed treatment with rhizobacterial isolates belonging to *Pseudomonas* and *Bacillus* group improved seedling growth in tomato (Anith, 2009). Improved seed colonization by the PGPR strains has been reported when coconut water was used as the medium for multiplication of the rhizobactera.

Tomato on inoculation with plant growth promoting rhizobacteria has been reported to contribute in enhancing plant growth regulators at the root interface, stimulating root development and thereby enhancing absorption of water and nutrients (Sharafzadeh, 2012).

Stress resistance and improved production of tomato was seen in seedlings treated with PGPR isolates (Almaghrabi *et al*., 2013). Meena *et al*. (2015) reported that when PGPR were used individualy and in combination with microorganism like *Azotobacter*, *Azospirillum* and *Actinomycetes,* enhanced yield of tomato has been realized.

Rhizosphere of tomato is colonized abundantly with *Bacillus* and *Pseudomonas* species which help in making phosphorous availablity to the plants (Lachisa and Dabassa, 2015). From the root zone of healthy tomato, five PGPR strains of different genera were isolated with high root colonizing capacity and phosphate solublization. The isolates were seen to promote the IAA production, with an enhanced nutrient uptake and chlorophyll content in the inoculated plant thereby increasing seed germination and seedling vigour (Babu *et al*., 2015).

An increased rate of IAA production was observed in tomato plants because of the presence of *Rhizobium* sp, *Pseudomonas* sp, *Bacillus* sp, *Agrobacterium*, etc. in the rhizosphere capable of producing IAA (Abbamondi *et al.*, 2016).

Pseudomonas sp RU47 increased phosphorus uptake and thereby improving the plant growth and promoting microbial phosphatase activity (PA) in the rhizosphere (Nassal *et al.*, 2018). Several strains of actinomycetes have the ability to produce high-value antibiotics, phytohormones, organic acids, bioactive compounds, extracellular enzymes and secondary metabolites. Other than their antimicrobial property, they help promote plant growth directly and indirectly (Singh *et al.*, 2018). *Bacillus* Bs10, Ba12 and Bl10 when added to the root zone of tomato help in improving the micro environment, increasing length, surface area and volume of tomato roots (Lou *et al.*, 2018). *Bacillus amyloliquefaciens* sub sp *plantarum* 32a exhibited plant-beneficial traits *in vitro* with an improvement in the growth of tomato plants by promoting germination and growth when compared to the untreated ones (Abdallah *et al.*, 2018).

2.4. CONSORTIUM INVOLVING *Piriformospora indica*

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 hike 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.* (2016) 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).

Materials and Methods

3. MATERIALS AND METHODS

The experiments envisaged in the research programme entitled "Management of bacterial wilt disease of tomato by the root endophytic fungus *Piriformospora indica*, rhizobacteria and bacterial endophytes" were carried out in the Department of Agricultural Microbiology, College of Agriculture, Vellayani during 2016-2018.

The details of materials used and methods employed in the study are described below.

3.1. THE BACTERIAL WILT PATHOGEN

3.1.1. Isolation of Bacterial Wilt Pathogen from Infected Plant Materials

Tomato plants (three numbers) showing typical bacterial wilt symptoms were collected from the fields of College of Agriculture, Vellayani, Thiruvananthapuram, Kerala. After thorough washing of root and collar region, the root system was cut and removed. A preliminary test for bacterial ooze was conducted with the stem portions. Plants which showed typical bacterial ooze were used for isolation of the bacterial wilt pathogen. Stem portion of the plants were cut into small bits of one cm length with a flame sterilized surgical knife. Further the bits were surface sterilized with one percent sodium hypochlorite aqueous solution for three minutes in a laminar air flow chamber. The bits were then washed with sterile distilled water thrice. They were transferred to a sterile Petri plate, crushed with a sterile glass rod and the ooze obtained was streaked on Semi selective agar medium from South Africa (SMSA) (Appendix I). The plates were incubated at 28°C ± 1°C for 48 h. Typical white fluidal colonies with red centre were selected for further purification by re-streaking on the SMSA (Schaad *et al.*, 2001). The bacterial isolates were stored on Casamino acid-Peptone-Glucose (CPG) slants under refrigerated condition for short term storage. Glycerol cultures were also maintained at -80°C.

4

3.1.2. Pathogenicity Test

The isolates obtained were inoculated separately to healthy 21 days old seedlings of tomato varieties Naveen and Vellayani Vijay procured from Department of Olericulture, College of Agriculture, Vellayani by soil drenching. A single colony from the CPG plate was picked and inoculated to CPG broth and incubated overnight in an incubator shaker (110 rpm) at 28°C ± 1°C. Inoculation with the bacterial isolate was done at the time of transplanting of tomato seedlings to the pots (15 cm dia) filled with unsterile soil. To the root zone of 10 plants each from both varieties, 10 ml each of the bacterial broth culture (10^{-7} cfu ml^{-1}) was applied. The plants were then observed daily for the incidence of bacterial wilt symptoms. Re-isolation of the pathogen was done from the infected plants as described above, following ooze test of the wilted plants. The most virulent isolate was further purified, stored both on slants and as glycerol culture for further studies.

3.1.3. Bacterial Growth Dynamics

Assessment of growth of the bacterial wilt pathogen in broth culture was carried out by standard plate count method and spectrophotometric (turbidimetric) analysis. The most virulent isolate stored on CPG slant was transferred to SMSA plates and incubated for 24 h at 28°C. A single colony from this was dispensed in 1 ml of sterile distilled water and mixed by vortexing briefly. Ten µl of inoculum was transferred to100 ml CPG broth in 250 ml flask and incubated in an incubator shaker (110 rpm, 28°C).

Serial dilution of the broth culture followed by spread plating on SMSA medium was done at 2 h intervals to assess the viable plate counts. Spectrophotometric measurement of the turbidity of broth was also done simultaneously and optical density (OD) value at 660nm was recorded. Finally a graph was plotted depicting OD Vs population of the bacterial growth.

Alternatively, the population assessment of the overnight grown bacterial culture was done by viable plate count method. The broth was then further diluted serially at 10 fold dilutions. OD value of the diluted samples at 660 nm was also recorded. The population data obtained from the undiluted overnight grown culture were extrapolated to fit to that of the diluted suspension. The data obtained were plotted to get a graph that shows OD Vs population.

3.2. MICROBIAL BIOAGENTS

3.2.1. Rhizobacteria

Bacillus pumilus VLY17, *Bacillus amyloliquefaciens* VLY24, *Pseudomonas fluorescens* PN026 and *Pseudomonas fluorescens* AMB8 were obtained from the Department of Agricultural Microbiology, College of Agriculture, Vellayani. The revived isolates of *Bacillus* and *Pseudomonas* strains from the glycerol cultures were routinely cultivated on Nutrient agar / Nutrient broth and King's medium B agar / King's medium B broth respectively at 28°C. The purified isolates were preserved on NA slants for further use.

3.2.2. Bacterial Endophytes

Rhizobium radiobacter PCRE10, *Bacillus velezensis* PCSE10, *Streptomyces leeuwenhoekii* KBT004 and *Bacillus megaterium* NAT001 were revived from previously preserved glycerol cultures available at the Department of Agricultural Microbiology, College of Agriculture, Vellayani. All isolates were cultivated on nutrient agar/ nutrient broth and incubated at 28°C. The purified isolates were preserved on NA slants for further use.

3.2.3. Fungal Endophyte

Piriformospora indica kindly provided by Dr. Ajit 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 was purified by the hyphal tip method on PDA plates and preserved on PDA slants under refrigeration.

The bioagents used in the present experiments are listed in table 1.

3.3. PLANT VARIETY

The plant varieties used were the bacterial wilt susceptible hybrid variety Naveen (Indo-American hybrid seeds Pvt. Ltd, Bengaluru) and the moderately wilt tolerant variety Vellayani Vijay (KAU, Department of Olericulture, College of Agriculture, Vellayani).

3.4. *In vitro* ANTAGONISM OF RHIZOBACTERIA AND BACTERIAL ENDOPHYTES AGAINST THE BACTERIAL WILT PATHOGEN

3.4.1. Direct Antagonism

Direct antagonism by the bacterial bioagents against bacterial wilt pathogen was assessed by cross streak plate assay, agar plug diffusion technique, disc diffusion method and spot on lawn method.

3.4.1.1. *Cross Streak Plate Assay*

A loopful of bacterial culture from a single colony of each of the test organisms was streaked separately across NA medium in a Petri plate as a straight line and incubated at 28°C. After 48 h *R. solanacearum* was streaked perpendicular to the test organism. The plates were further incubated at 28°C for 48 h. Three replications were maintained. Antagonistic interaction was noticed by measuring the inhibition zone between the test organism and pathogen at the site of cross streak (Balouiri *et al.*, 2016).

Table 1. Bioagents used in the present study

Sl. No.	Organisms	Source	Reference
1	*Piriformospora indica*	Dr. Ajit Varma Professor, Jawaharlal Nehru University, New Delhi.	Varma *et al*., 1999
2	*Bacillus amyloliquefaciens* VLY24	Department of Agricultural Microbiology, COA, Vellayani.	Varkey *et al*., 2018
3	*B. pumilus* VLY17	Department of Agricultural Microbiology, COA, Vellayani.	Varkey *et al*., 2018
4	*Pseudomonas fluorescens* PN026	Department of Agricultural Microbiology, COA, Vellayani.	Anith *et al*., 2002
5	*P. fluorescens* AMB8	Department of Agricultural Microbiology, COA, Vellayani.	Anith *et al*., 2002
6	*R. radiobacter* PCRE10	Department of Agricultural Microbiology, COA, Vellayani.	Kollakkodan *et al*., 2017
7	*B. velezensis* PCSE10	Department of Agricultural Microbiology, COA, Vellayani.	Kollakkodan *et al*., 2017
8	*B. megaterium* NAT001	Department of Agricultural Microbiology, COA, Vellayani.	Vyshakhi, 2016
9	*S. leeuwenhoekii* KBT004	Department of Agricultural Microbiology, COA, Vellayani.	Vyshakhi, 2016

3.4.1.2. *Agar Plug Diffusion Technique*

Heavy cross streaking of *Pseudomonas* strains was done on King's medium B agar plates and all the other bacterial strains on nutrient agar medium. The plates were incubated at 28°C for 24 h. *R. solanacearum* was swab inoculated uniformly over nutrient agar plates. Using a cork borer, agar discs (5 mm) from the cross streaked plates were cut out and placed over the Petri plate containing *R. solanacearum* at four different corners equidistantly. For each bacterial isolate, three replications were maintained. The inoculated plates were incubated at 28°C for 48 h. Antagonistic interaction between the test organism and pathogen was noticed by measuring the inhibition zone around the agar plug (Balouiri *et al.*, 2016).

3.4.1.2. *Disc Diffusion Method*

R. solanacearum was swab inoculated on Nutrient agar plates covering the entire surface uniformly. *Pseudomonas* strains were inoculated in King's B broth and all the other bacterial strains in nutrient broth and incubated at 28°C for 24 h in an incubator shaker (110 rpm). Ten µl each of the bacterial suspension was transferred to sterile filter paper discs (5 mm dia). The discs were dried in a laminar air flow chamber and placed on the agar plate swabbed with *R. solanacearum*. Three replications were maintained for each of the bacterial isolate. Plates were then incubated for a period of 48 h in 28°C. Antagonistic interaction between the test organism and the pathogen was noticed by measuring the inhibition zone around the discs (Nawangsih *et al.*, 2011).

3.4.1.3. *Spot on Lawn Method*

Swab inoculation of *R. solanacearum* was done on Nutrient agar plates. *Pseudomonas* strains were inoculated in King's B broth and all the other bacterial strains were inoculated in nutrient broth and incubated at 28°C for 24 h in an incubator shaker. Twenty µl each of the overnight grown test organisms was spotted

on the swab inoculated plate at four corners using a micropipette. Three replications were maintained for each of the bacterial isolate. Plates were incubated for a period of 48h at 28°C. Antagonistic interaction between the test organism and the pathogen was noticed by measuring the inhibition zone around the spot (Cao *et al*., 2018).

3.4.2. Indirect Antagonism

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

3.4.2.1. Antagonism by Culture Filtrate of Bacterial Endophytes, Rhizobacteria and Fungal Endophyte Against the Bacterial Wilt Pathogen

3.4.2.1.1. 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 or nutrient broth for *Pseudomonas* strains and other bacterial isolates respectively. Cultures were incubated overnight in an 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.

A single mycelial plug (8 mm dia) of *P. indica* grown on PDA plate was cut out from the leading edge of the growth and transferred to 100 ml of Potato Dextrose broth (PDB) taken in a 250 ml conical flask. It was incubated for seven days in an incubator shaker (110 rpm) at 28°C. The mycelial growth was removed by straining through a muslin cloth under aseptic conditions. Ten ml of the liquid portion was centrifuged at 10,000 rpm for five minutes in a 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.

3.4.2.1.2. Agar Well Diffusion Method

R. solanacearum was swab inoculated on nutrient agar plates. Wells (8 mm dia) were cut at four corners of the plate using a sterile cork borer. The wells were partially filled with 100 μl of molten nutrient agar. Once the well was sealed properly, 200 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 diameter of inhibition zone around the well was measured (Balouiri *et al.*, 2016).

3.4.2.1.3. Disc Diffusion Method

Swab inoculation of *R. solanacearum* was carried out on nutrient agar plates. Ten μl each of the culture filtrate was transferred to sterile filter paper discs (5 mm dia). The discs were dried in a laminar air flow chamber and placed at four corners of the Petri plate containing the pathogen. Plates were then incubated for a period of 48 h at 28°C. Three replications were maintained for each of the organism. Inhibition zone around the filter paper disc was measured (Nawangsih *et al.*, 2011).

3.5. COMPATIBILITY ASSSESMENT OF BACTERIAL BIOAGENTS WITH *P.indica* BY DUAL CULTURE PLATE ASSAY

Single colonies of *Pseudomonas* strains and other bacterial strains were obtained by streak plating on King's medium B agar or Nutrient Agar respectively. 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. When the fungal growth reached a diameter of 5 cm, each of the test organisms was streaked as a band (5 cm)

separately on two sides of the PDA plate 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).

3.6. CO-CULTURING OF COMPATIBLE BACTERIAL AGENT WITH *P.indica*

One hundred ml each of PDB (pH of 6.5) was sterilized in 250 ml Erlenmeyer flask and inoculated with two mycelial plugs (8 mm dia) of *Piriformospora indica* obtained from PDA plates previously grown for 10 days. Bacterial isolates were streaked on nutrient agar medium for obtaining single colonies. One loopful of the test organism was mixed in 1 ml of sterile distilled water and 20 μl of the bacterial suspension was transferred to the conical flask containing *P. indica* growth. Bacterial suspension was also inoculated to PDB without *P. indica* growth. Inoculated flasks were incubated in an incubator shaker (110 rpm) at 28°C for 24 h. Bacterial growth was assessed by viable plate count at 0 and 24 h of incubation, both from the co-cultured flask and from flasks wherein bacterial cultures were grown independently (Anith *et al.*, 2015).

3.7. *IN VIVO* EXPERIMENTS

3.7.1. *In vivo* Evaluation of Bioagents for the Suppression of Bacterial Wilt Incidence

3.7.1.1. *Nursery Production of Tomato Plants*

Potting mixture was prepared by mixing vermiculite and perlite in the ratio of 2:1. 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 tomato 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 and all the other bacterial strains on nutrient agar medium. 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 respective 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 3.4.2.1.1) 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 @ 1 per cent (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 per cent shade. Seedlings were irrigated with tap water twice daily. Once in 10 days, fertigation was provided by pouring 10 ml of 1 per cent water soluble fertilizer solution (N:P:K - 17:17:17) per cavity starting from first week after seeding. Plants were kept for 21 days in the nursery (Plate 1). Separate nurseries were raised for the tomato varieties Naveen and Vellayani Vijay.

Plate 1. Nursery production of tomato plants in pro-tray cavities

Plate 2. General view of the experimental plot

3.7.1.2. ***Suppression of Bacterial Wilt Incidence in Variety Naveen by Application of Individual Bioagents***

Seedlings of the bacterial wilt susceptible variety Naveen were raised as described in section 3.7.1.1. The following treatments were imposed during the nursery production.

T_1: *Bacillus pumilus* VLY17

T_2: *B. amyloliquefaciens* VLY24

T_3: *Pseudomonas fluorescens* PN026

T_4: *P. fluorescens* AMB8

T_5: *B. velezensis* PCSE10

T_6: *Rhizobium radiobacter* PCRE10

T_7: *Streptomyces leeuwenhoekii* KBT004

T_8: *B. megaterium* NAT001

T_9: *Piriformospora indica*

T_{10}: Inoculated control

T_{11}: Absolute control

Design : CRD

Treatments : 11

Replications : 3

Number of plants per replication: 5

3.7.1.2.1. *Transplanting of the Seedlings and Challenge Inoculation*

Twenty one day-old seedlings were transplanted to pots (15 cm dia) filled with one kg each of garden soil (Plate 2). Five days after transplanting, challenge inoculation with the bacterial wilt pathogen was performed. Single colonies of highly virulent *R. solanacearum* from the CPG plates were transferred to CPG broth and incubated in an incubator shaker overnight (110 rpm) at 28°C. 10 ml of the overnight grown bacterial culture adjusted to 10^7cfu ml^{-1} was poured on the root zone of each plant for challenge inoculation.

Observation on bacterial wilt disease incidence was recorded at weekly intervals from the day of challenge inoculation.

% Disease incidence calculated by the formula;

$$\% \text{ Disease incidence} = \frac{\text{Number of wilted plants}}{\text{Total number of plants}} \times 100$$

3.7.1.3. *Suppression of Bacterial Wilt Incidence in Variety Naveen*

Seedlings of the bacterial wilt susceptible variety Naveen were raised as described in section 3.7.1.1. In the case of mixed inoculation, the fungal endophyte was mixed with planting medium prior to filling the pro-trays and bacterized seeds were planted into pro-trays filled with *P. indica* incorporated planting medium. The following treatments were imposed during the nursery production.

T_1- *Piriformospora indica* + *Bacillus amyloliquefaciens* VLY24

T_2- *P. indica* + *Streptomyces leeuwenhoekii* KBT004

T_3- *P. indica* + *B. velezensis* PCSE10

T_4- *P. indica* + *Rhizobium radiobacter* PCRE10

T_5- Inoculated control

T_6- Absolute control

Design : CRD

Treatments : 6

Replications : 3

Number of plants per replication: 5

3.7.1.3.1. ***Transplanting of the Seedlings and Challenge Inoculation***

Transplanting of the seedlings and challenge inoculation was done as described in section 3.7.1.2.1.

3.7.1.4. ***Root Colonization by Piriformospora indica***

The surviving plants treated with *Piriformospora indica* were assessed for root colonization by the endophytic fungus. 21 days after plant growth, five plants 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;

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

3.7.2.1. ***Suppression of Bacterial Wilt Incidence in Variety Vellayani Vijay by Application of Individual Bioagents***

Seedlings of the moderately wilt tolerant variety Vellayani Vijay were raised as described in section 3.7.1.1. The treatments imposed during nursery production were same as that described in section 3.7.1.2.

3.7.2.2. ***Nursery Production of Tomato Plants***

Nursery production of tomato plants was done as described in section 3.7.1.1.

3.7.2.2.1. ***Transplanting of the Seedlings and Challenge Inoculation***

Transplanting of the seedlings and challenge inoculation was done as described in section 3.7.1.2.1.

3.7.2.3. ***Suppression of Bacterial Wilt Incidence in Variety Vellayani Vijay***

Seedlings of the moderately wilt tolerant variety Vellayani Vijay were raised as described in section 3.7.1.1. The treatments imposed during nursery production were same as that in section 3.7.2.2.

3.7.2.3.1. ***Transplanting of the Seedlings and Challenge Inoculation***

Transplanting of the seedlings and challenge inoculation was done as described in section 3.7.1.2.1.

3.8. PLANT GROWTH PROMOTION BY RHIZOBACTERIA, BACTERIAL ENDOPHYTE AND FUNGAL ENDOPHYTE IN VARIETY VELLAYANI VIJAY

3.8.1. Nursery Production of Tomato Plants

Nursery production of tomato plants was done as described in section 3.7.1.1. The plants were kept for 30 days in the nursery after the following treatments were imposed.

T_1- *Bacillus amyloliquefaciens* VLY24

T_2- *B. pumilus* VLY17

T_3- *Pseudomonas fluorescens* PN026

T_4- *P. fluorescens* AMB8

T_5- *B. velezensis* PCSE10

T_6- *Rhizobium radiobacter* PCRE10

T_7- *Streptomyces leeuwenhoekii* KBT004

T_8- *B. megaterium* NAT001

T_9- *Piriformospora indica*

T_{10}- *P. indica+ B. amyloliquefaciens* VLY24

T_{11}- *P. indica + S. leeuwenhoekii* KBT004

T_{12}- *P. indica + B.. velezensis* PCSE10

T_{13}- *P. indica + R. radiobacte r*PCRE10

T_{14}- Control

Design: CRD

Treatments: 15

Replications: 3

Number of plants per replication: 5

3.8.2. Biometric Observation

Thirty days after seeding, biometric observations were taken following destructive sampling. The observations recorded were: number of leaves plant^{-1}, plant height (cm), fresh root weight (g), fresh shoot weight (g), dry root weight (mg) and dry shoot weight (mg). The dry weights were taken after drying plant samples for three days in 60°C in a hot air oven.

3.9. 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 Duncan's Multiple Range Test (DMRT).

Results

4. RESULTS

The data obtained under the present investigation on "Management of bacterial wilt disease of tomato by the root endophytic fungus *Piriformospora indica*, rhizobacteria and bacterial endophytes" were analyzed and the results obtained are presented in this chapter under following headings.

4.1. THE BACTERIAL WILT PATHOGEN

4.1.1. Isolation of Bacterial Wilt Pathogen from Infected Plant Materials

Ralstonia solanacearum, the pathogen causing bacterial wilt disease in tomato, was isolated from the stem portions of the infected tomato plants showing typical wilt symptoms (Plate 3). The samples were collected from different tomato growing fields of College of Agriculture, Vellayani and tested for the presence of ooze (Plate 4). The plants showing positive results in ooze test were further used for the isolation of the pathogen.

On SMSA plates, white fluidal colonies with red centre were obtained. The colonies tend to stream across the plate to the lid and to the extreme ends of the plates (Plate 5). Two isolates of *Ralstonia solanacearum* were obtained from which the best isolate was selected based on the pathogenicity test.

The isolates obtained were further purified by quadrant streaking and cultures derived from single colonies were stored on CPG slants and as glycerol cultures.

4.1.2. Pathogenicity Test

The pathogenicity of the isolates obtained was confirmed by proving Koch's postulates. The isolated pathogen was artificially inoculated on 21 days old seedlings of the tomato varieties, Naveen and Vellayani Vijay. The symptoms appeared were same as that observed in the plants from which the isolates were obtained. The plants

Plate 3. Tomato plants with typical bacterial wilt symptoms in the field

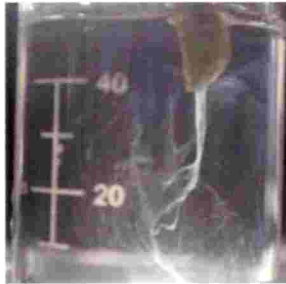


Plate 4. Bacterial ooze from the stem portions of infected plants

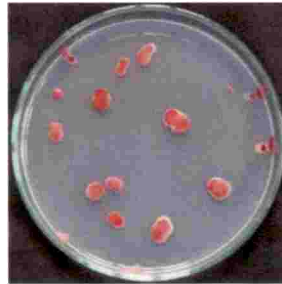


Plate 5. Colonies of *Ralstonia solanacearum* on SMSA agar medium

Plate 6. Wilt symptom in tomato plant artificially inoculated with virulent strain of *Ralstonia solanacearum*

exhibited sudden wilting even when they stood green; leaves turned flaccid and several adventitious roots were produced at the base of the stem. Wilting occurred during the hottest period of day which recovered in the evening hours (Plate 6). The wilted plants were further tested for ooze and those showing positive results were used for re-isolation of the pathogen. The re-isolated pathogen was identical to the original isolate in all morphological and cultural characters. Most virulent isolate was selected based on the intensity of disease produced and further purified by quadrant streaking and stored on slants and as glycerol culture.

4.1.3. Bacterial Growth Dynamics

The most virulent isolate obtained from the pathogenicity test was grown in CPG broth and a functional relationship between the population and its optical density (OD) at 660 nm was worked out for further use. The data obtained are presented in table 2 and 3. The graphical relationship between population and OD values is depicted in figure 1 and 2. Inoculum density for challenge inoculation with the pathogen in the biocontrol experiments was determined based on the OD value of the broth culture.

4.2. *In vitro* ANTAGONISM OF RHIZOBACTERIA AND BACTERIAL ENDOPHYTES AGAINST THE BACTERIAL WILT PATHOGEN

4.2.1. Direct Antagonism

4.2.1.1. *Cross Streak Plate Assay*

The observations on antagonistic effect of four bacterial endophytes and four rhizobacterial isolates tested against *R. solanacearum* are given in table 4. The results of the cross streak plate assay showed that *Bacillus amyloliquefaciens* VLY24, *B. velezensis* PCSE10 and *Streptomyces leeuwenhoekii* KBT004 exhibited antibacterial activity against the wilt pathogen evidenced by presence of inhibition zone (Plate 7). Zone of inhibition was the largest for *B. amyloliquefaciens* VLY24 (2.0 cm) followed

Table 2. Population of *Ralstonia solanacearum* in CPG broth and OD value at 660 nm measured at 2 h interval.

Time of observation	Population (log cfu/ml)*	OD value
0 hour	7.102777	0.054
2 hour	7.598462	0.14
4 hour	8.522835	0.298
6 hour	8.623249	0.487
8 hour	8.740363	0.79

*Mean of 3 replications

Table 3. Population of *Ralstonia solanacearum* in the serially diluted samples from overnight grown CPG broth and OD value at 660 nm.

Dilution	Population (log cfu/ml)*	OD value
Undiluted	9.5769	1.7685
10^{-1}	8.5769	0.286
10^{-2}	7.5769	0.0335
10^{-3}	6.5769	0.004
10^{-4}	5.5769	0.0025

* Mean of 3 replications

Table 4. Interaction of bacterial bioagents and *Ralstonia solanacearum* assessed by cross streak plate assay

Bacterial bioagents	**Growth inhibition**	**Inhibition zone (cm)***
Bacillus amyloliquefaciens VLY24	+	2.0
B. pumilus VLY17	-	Nil
Pseudomonas fluorescens PN026	-	Nil
P. fluorescens AMB8	-	Nil
Rhizobium radiobacter PCRE10	-	Nil
B. velezensis PCSE10	+	1.4
B. megaterium NAT001	-	Nil
Streptomyces leeuwenhoekii KBT004	+	0.4

* Mean of three replications

+ Antagonism present

- Antagonism absent

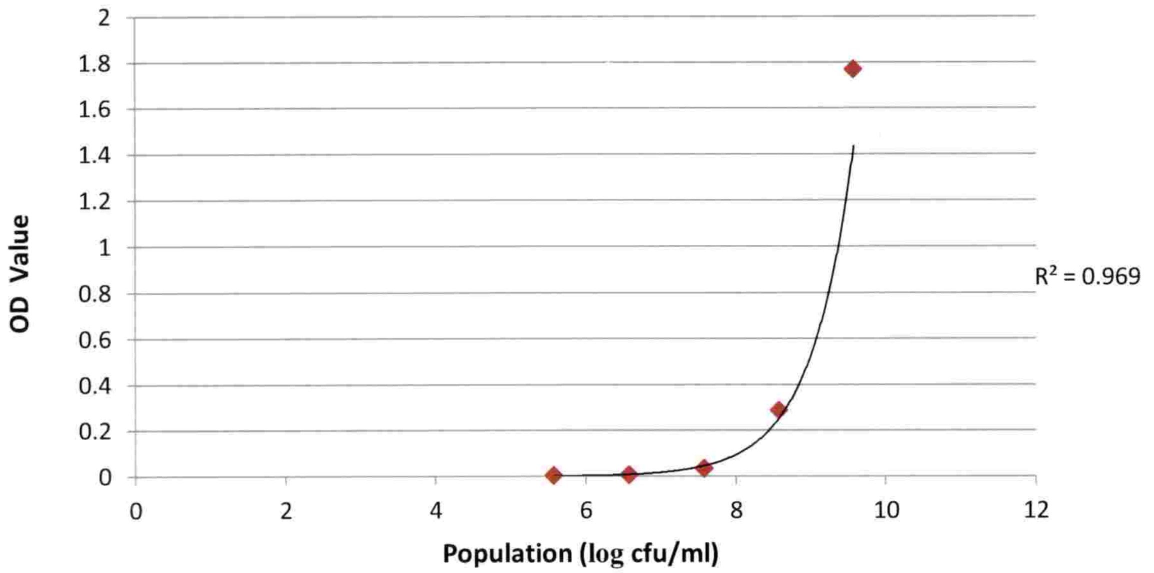


Figure 1. Relationship between optical density and population of *R. solanacearum* in batch culture system

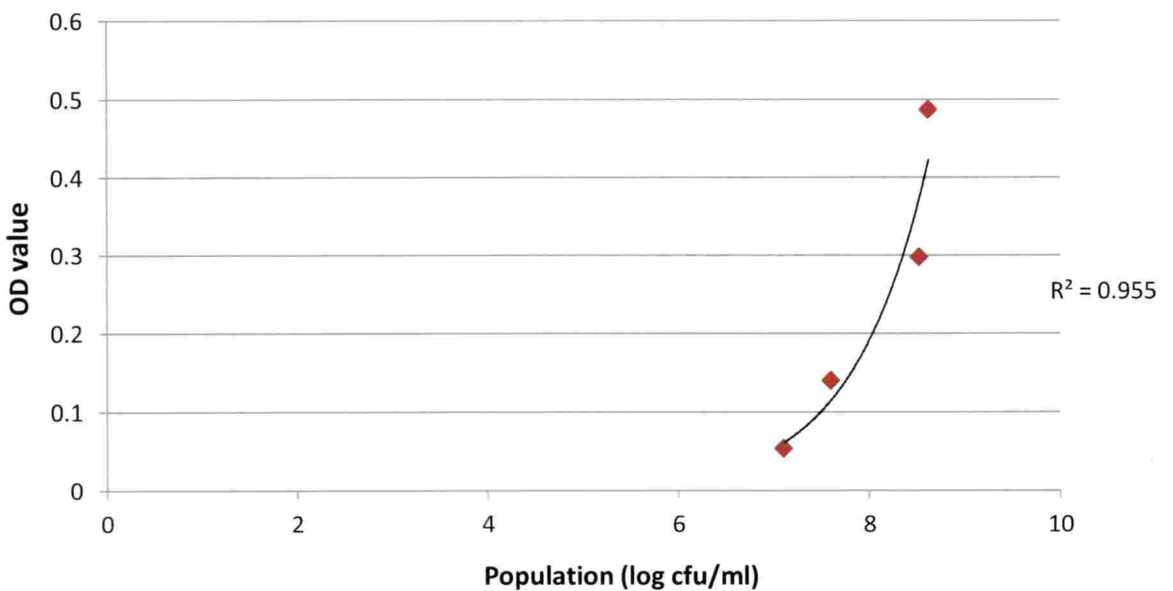
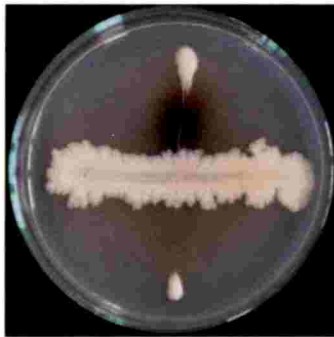
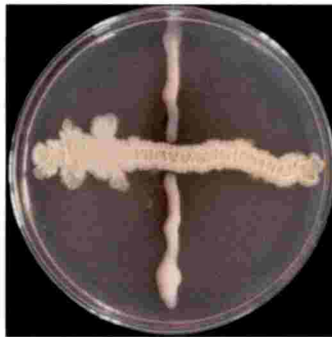


Figure 2. Relationship between the optical density and population of *R. solanacearum* in alternate assay



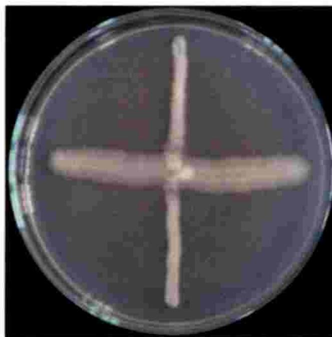
Ba VLY24



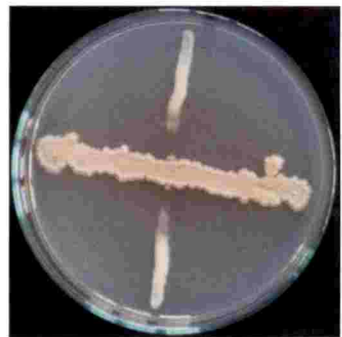
Bp VLY17

Pf PN026

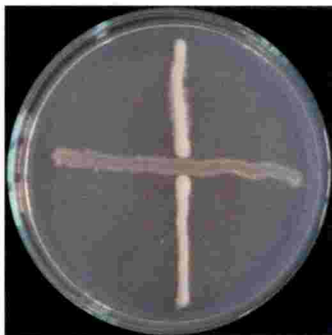
Pf AMB8



Rr PCRE10



Bv PCSE10



Bm NAT001

Sl KBT004

Plate 7. Cross streak plate assay for checking antagonism between bacterial bioagents and *Ralstonia solanacearum*

Ba- *Bacillus amyloliquefaciens,* Bp- *B. pumilus,* Pf- *Pseudomonas fluorescens,* Rr- *Rhizobium radiobacter,* Bv- *B. velezensis,* Bm- *B. megaterium,* Sl- *Streptomyces leeuwenhoekii*

by that produced in *B. velezensis* PCSE10 (1.4 cm) and *S. leeuwenhoekii* KBT004 (0.4 cm). Other bacterial isolates showed no inhibition of the growth of the pathogen.

4.2.1.2. *Agar Plug Diffusion Technique*

When the bacterial bioagents were tested against *Ralstonia solanacearum* by agar plug diffusion technique, it was observed that *Bacillus amyloliquefaciens* VLY24, *B. velezensis* PCSE10 and *S. leeuwenhoekii* KBT004 produced inhibition zone against the pathogen (Plate 8A). Inhibition zone was highest for *B. amyloliquefaciens* VLY24 followed by *B. velezensis* PCSE10 and *S. leeuwenhoekii* KBT004. No other bacterial isolates showed any inhibition to the growth of the pathogen (Table 5).

4.2.1.3. *Disc Diffusion Method*

In the disc diffusion method, zone of inhibition around the discs containing *Bacillus amyloliquefaciens* VLY24, *B. velezensis* PCSE10 and *Streptomyces leeuwenhoekii* KBT004 was observed (Plate 8B, Table 6). Other bacterial isolates did not produce any inhibition against *Ralstonia solanacearum.*

4.2.1.4. *Spot on Lawn Method*

Zone of inhibition against *Ralstonia solanacearum* was observed around the spot containing *Bacillus amyloliquefaciens* VLY24, *B. velezensi* PCSE10 and *Streptomyces leeuwenhoekii* KBT004 (Plate 8C). The presence or absence of inhibition zone is presented in table 7.

4.4.2. Indirect Antagonism

4.4.2.1. *Antagonism by Culture Filtrate of Bacterial Endophytes, Rhizobacteria and Fungal Endophyte Against Bacterial Wilt Pathogen*

Table 5. Interaction of bacterial bioagents and *Ralstonia solanacearum* assessed by agar plug diffusion technique

Bacterial bioagents	**Growth inhibition**
Bacillus amyloliquefaciens VLY24	+
B. pumilus VLY17	-
Pseudomonas fluorescens PN026	-
P. fluorescens AMB8	-
Rhizobium radiobacter PCRE10	-
B. velezensis PCSE10	+
B. megaterium NAT001	-
Streptomyces leeuwenhoekii KBT004	+

+ Antagonism present

- Antagonism absent

Table 6. Interaction of bacterial bioagents and *Ralstonia solanacearum* assessed by disc diffusion methods

Bacterial bioagents	Growth inhibition
Bacillusamyloliquefaciens VLY24	+
B. pumilus VLY17	-
Pseudomonas fluorescens PN026	-
P. fluorescens AMB8	-
Rhizobium radiobacter PCRE10	-
B. velezensis PCSE10	+
B. megaterium NAT001	-
Streptomyces leeuwenhoekii KBT004	+

\+ Antagonism present

\- Antagonism absent

Table 7. Interaction of bacterial bioagents and *Ralstonia solanacearum* assessed by spot on lawn method

Bacterial bioagents	Growth inhibition
Bacillus amyloliquefaciens VLY24	+
B. pumilus VLY17	-
Pseudomonas fluorescens PN026	-
P. fluorescens AMB8	-
Rhizobium radiobacter PCRE10	-
B. velezensis PCSE10	+
B. megaterium NAT001	-
Streptomyces leeuwenhoekii KBT004	+

+ Antagonism present

- Antagonism absent

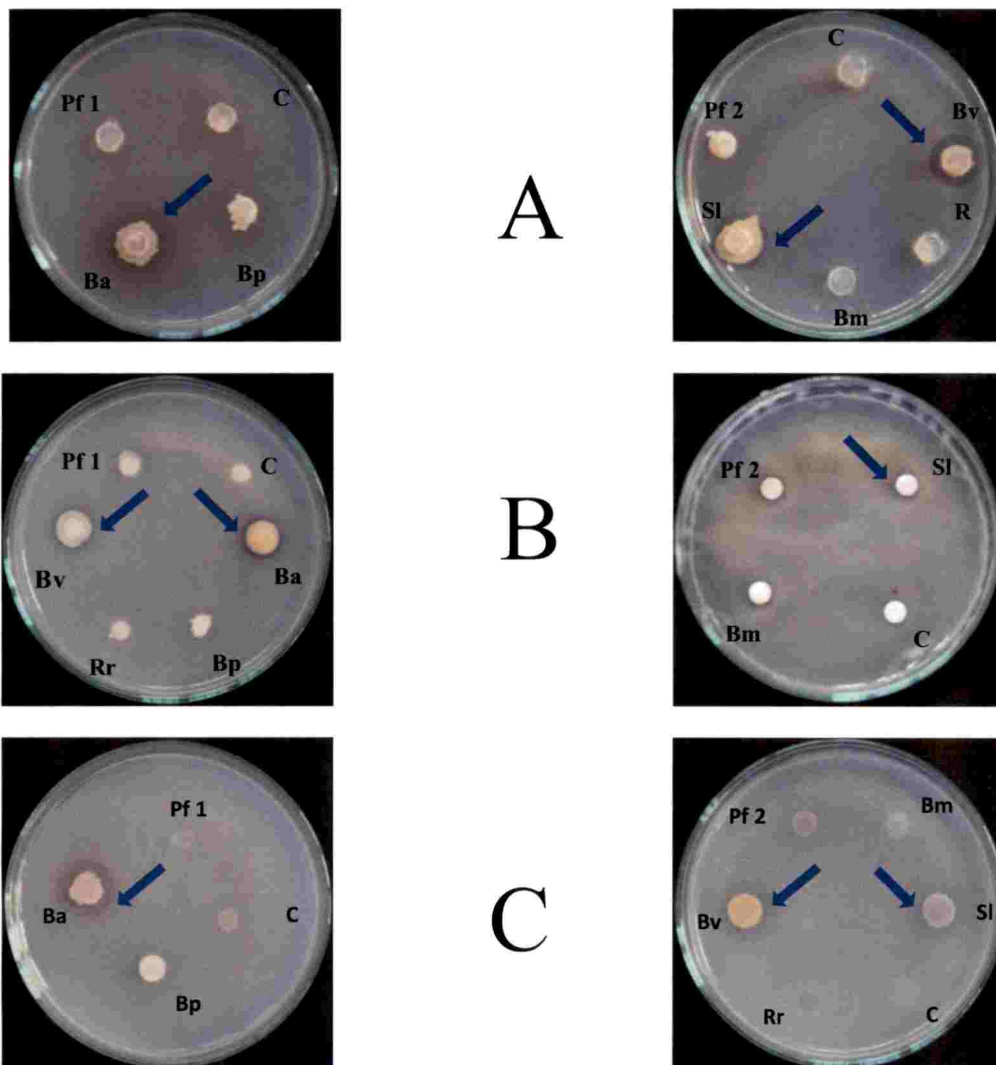


Plate 8. Direct methods for checking antagonism between bacterial bioagents and *Ralstonia solanacearum* A- Agar plug diffusion method; B- Disc diffusion method; C – Spot on lawn method

Ba- *Bacillus amyloliquefaciens* VLY24, Bp- *B. pumilus* VLY17, Pf 1- *Pseudomonas fluorescens* PN026, Pf 2- *P. fluorescens* AMB8, Rr- *Rhizobium radiobacter* PCRE10, Bv- *B. velezensis* PCSE10, Bm- *B. megaterium* NAT001, Sl- *Streptomyces leeuwenhoekii* KBT004, C- Control

4.4.2.1.1. *Agar Well Diffusion Method*

The culture filtrates of *Bacillus velezensis* PCSE10, *B. amyloliquefaciens* VLY24 and *Piriformospora indica* exhibited antibacterial activity against the pathogen. Zone of inhibition was the highest for *B. velezensis* PCSE10 (0.7 cm) followed by that for *B. amyloliquefaciens* VLY24 (0.4 cm) and *P. indica* (0.1 cm) (Table 8; Plate 9A). Cell free extracts of other bacterial isolates showed no inhibition on growth of the pathogen.

4.4.2.1.2. *Disc Diffusion Method*

The discs impregnated with culture filtrate of *Bacillus velezensis* PCSE10, *B. amyloliquefaciens* VLY24 and *Piriformospora indica* exhibited antagonistic activity against the pathogen by producing inhibition zone (Plate 9B). Zone of inhibition was maximum for *B. velezensis* PCSE10 (0.3 cm) and was followed by *B. amyloliquefaciens* VLY24 (0.1 cm) and *P. indica* (0.1 cm) (Table 9). Cell free extracts of other bacterial isolates did not have any inhibition on the growth of the pathogen.

4.5. COMPATIBILITY OF BACTERIAL BIOAGENTS WITH *Piriformospora indica*

In vitro interaction of bacterial bioagents and *Piriformospora indica* using dual culture plate assay was done to assess the compatibility between them (Plate 10). The results showed that *P. indica* growth was not inhibited by *Rhizobium radiobacter* PCRE10, *Bacillus megaterium* NAT001 and *Streptomyces leeuwenhoekii* KBT004. However, *Pseudomonas fluorescens* PN026 and *P. fluorescens* AMB8 produced a zone of inhibition of 0.5 mm each. *B. velezensis* PCSE10 and *B. amyloliquefaciens* VLY24 produced an inhibition zone of 0.4 mm and *B. pumilus* VLY17 produced an inhibition zone of 0.1 mm (Table 10).

Table 8. Effect of the culture filtrate of bioagents on *Ralstonia solanacearum* assessed by agar well assay

Bacterial bioagents	**Growth inhibition**	**Inhibition zone (cm)***
Bacillus amyloliquefaciens VLY24	+	0.4
B. pumilus VLY17	-	Nil
Pseudomonas fluoresens PN026	-	Nil
P. fluoresens AMB8	-	Nil
Rhizobium radiobacter PCRE10	-	Nil
B. velezensis PCSE10	+	0.7
B. megaterium NAT001	-	Nil
Streptomyces leeuwenhoekii KBT004	-	Nil
Piriformospora indica	+	0.1

* Mean of three replications

+ Antagonism present

- Antagonism absent

Table 9. Effect of the culture filtrate of bioagents on *Ralstonia solanacearum* assessed by disc diffusion method

Bacterial bioagents	Growth inhibition	Inhibition zone (cm)*
Bacillus amyloliquefaciens VLY24	+	0.1
B. pumilus VLY17	-	Nil
Pseudomonas fluorescens PN026	-	Nil
P. fluorescens AMB8	-	Nil
Rhizobium radiobacter PCRE10	-	Nil
B. velezensis PCSE10	+	0.3
B. megaterium NAT001	-	Nil
Streptomyces leeuwenhoekii KBT004	-	Nil
Piriformospora indica	+	0.1

* Mean of three replications

\+ Antagonism present

\- Antagonism absent

Table 10. *In vitro* assessment for compatability of bacterial bioagents with *Piriformospora indica* by dual culture plate assay

Bacterial bioagents	**Growth inhibition**	**Inhibition zone (cm)***
Bacillus amyloliquefaciens VLY24	+	0.4
B. pumilus VLY17	+	0.1
Pseudomonas fluorescens PN026	+	0.5
P. fluorescens AMB8	+	0.5
Rhizobium radiobacter PCRE10	-	Nil
B. velezensis PCSE10	+	0.4
B. megaterium NAT001	-	Nil
Streptomyces leeuwenhoekii KBT004	-	Nil

* Mean of three replications

\+ Antagonism present

\- Antagonism absent

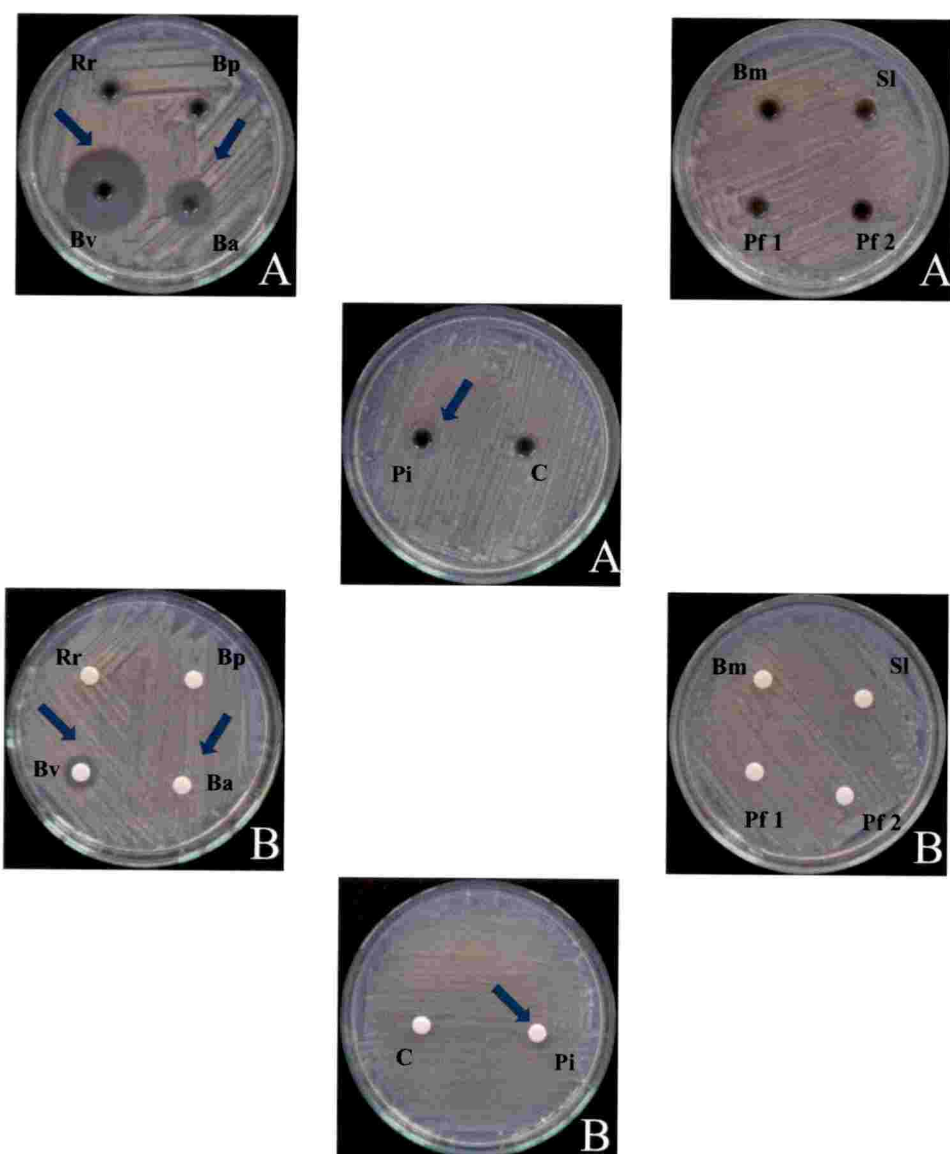
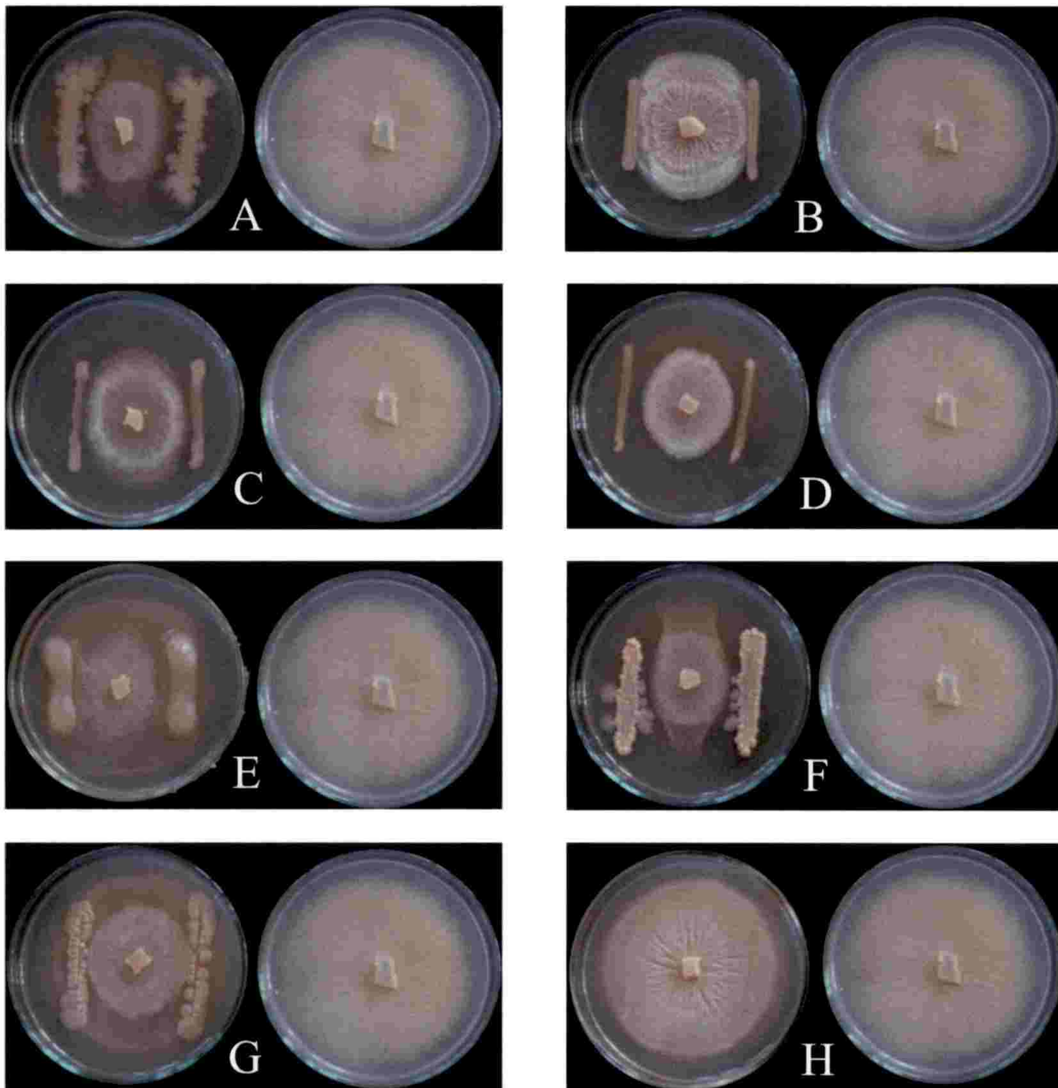


Plate 9. Indirect methods used for checking antagonism of bacterial bioagents and the fungal endophyte against *Ralstonia solanacearum* A. Agar well diffusion method; B. Disc diffusion method

Ba- *Bacillus amyloliquefaciens* VLY24, Bp- *B. pumilus* VLY17, Pf 1- *Pseudomonas fluorescens* PN026, Pf 2 – *P. fluorescens* AMB8, Rr- *Rhizobium radiobacter* PCRE10, Bv- *B. velezensis* PCSE10, Bm- *B. megaterium* NAT001, Sl- *Streptomyces leeuwenhoekii* KBT004, Pi- *Piriformospora indica*



Plates 10. ***In vitro*** **assessment of compatibility between bacterial bioagents and *Piriformospora indica* by dual culture plate assay**

A-*Bacillus amyloliquefaciens* VLY24, B- *B. pumilus* VLY17, C- *Pseudomonas fluorescens* PN026, D- *P. fluorescens* AMB8, E- *Rhizobium radiobacter* PCRE10, F- *B. velezensis* PCSE10, G- *B. megaterium* NAT001, H- *Streptomyces leeuwenhoekii* KBT004.

4.6. CO-CULTURING OF COMPATIBLE BACTERIAL AGENT WITH *P. indica*

Co-culturing of the bacterial bioagents with the endophytic fungus *Piriformospora indica* in PDB showed varying levels of population build up of the bacteria after 24 h of incubation (Table 11). When grown individually in PDB all the bacterial isolates were able to multiply by several fold. Increase in the level of population build up in the co-culture system was observed with the bacterial strains *Rhizobium radiobacter* PCRE10 and *Streptomyces leeuwenhoekii* KBT004. No increase in population of *Bacillus megaterium* NAT001was observed during the co-culture as dilution plating either at a level that corresponded to the initial population or any dilution higher than that revealed no presence of colonies in the plates.

4.7. *In vivo* EXPERIMENTS

4.7.1. *In vivo* Evaluation of Bioagents for the Suppression of Bacterial Wilt Incidence in Variety Naveen

4.7.1.1. *Suppression of Bacterial Wilt Incidence by Application of Individual Bioagents*

Bacterial wilt incidence was recorded at weekly intervals after challenge inoculation with the pathogen. The data thus obtained were further processed for finding Percentage Disease Incidence (PDI). Observation on disease incidence from 7th day, 14th day and 21stday were statistically analyzed.

Seven days after inoculation, the minimum disease incidence was noticed in the plants treated with *R. radiobacter* PCRE10 which was statistically on par with the absolute control. *B. velezensis* PCSE10 and *P. indica* also exhibited a significant reduction in the level of disease incidence which was also on par with the above mentioned treatments. Plants treated with both strains of *P. fluorescens* showed high

Table 11. Population assessment of bacterial bioagents during co-culturing with *Piriformospora indica*

Treatment	Population (log cfu/ml)	
	Initial (0th time)	Final (24 h)
Rhizobium radiobacter PCRE10	4.97	9.27
R. radiobacter PCRE10 + *P. indica*	4.71	5.10
Bacillus megaterium NAT001	4.17	5.70
B. megaterium NAT001 + *P. indica*	4.14	ND
Streptomyces leeuwenhoekii KBT004	4.34	5.80
S. leeuwenhoekii KBT004 + *P. indica*	3.00	5.85

ND – Not detected any number of colonies at a dilution that corresponded to the initial population.

levels of disease incidence and was on par with the inoculated control having a disease incidence of 25 per cent (Table 12).

On the 14th day after inoculation, disease incidence was scored minimum in plants treated with *R. radiobacter* PCRE10 which was on par with those treated with *B. velezensis* PCSE10 and *P. indica.* High level of disease incidence was noticed in plants treated with *P. fluorescens* AMB8 (50 percent) followed by *S. leeuwenhoekii* KBT004 and *P. fluorescens* PN026 (40 percent each) (Table 13).

On the 21st day after inoculation, plants treated with *R. radiobacter* PCRE10 recorded significantly lesser disease incidence which was on par with those treated with *B. velezensis* PCSE10. The plants that received treatment with *P. fluorescens* AMB8 showed high level of disease incidence followed by those treated with *B. pumilus* VLY17, *P. fluorescens* PN026, *B. megaterium* NAT001, *S. leeuwenhoekii* KBT004 and *P. indica* (Table 14).

4.7.1.2. *Suppression of Bacterial Wilt Incidence by Combined Application of Bioagents*

After one week of challenge inoculation, no disease incidence was observed in the plants treated with paired combination of *P. indica* with *B. amyloliquefaciens* VLY24 and *S. leeuwenhoekii* KBT004. Mixed inoculation of *P. indica* with *R. radiobacter* PCRE10 exhibited high level of disease incidence (30 per cent) which was on a par with that in the inoculated control (Table 15).

On the 14th day after inoculation, disease incidence was not noticed in the plants treated with combination of *P. indica* and *S. leeuwenhoekii* KBT004 which was on par with the combined application of *P. indica* and *B. amyloliquefaciens* VLY24 (5.0 percent). *P. indica* when applied in combination with *R. radiobacter* PCRE10 showed high level of disease incidence (55 percent) as in the previous scoring week (Table 16).

Table 12. Incidence of bacterial wilt in the variety Naveen observed 7 days after challenge inoculation

Treatment	**Percentage Disease Incidence***
Bacillus amyloliquefaciens VLY24	10.00 (16.49)[cb]
B. pumilus VLY17	15.00 (21.53)[dc]
Pseudomonas fluorescens PN026	30.00 (32.90)[fe]
P. fluorescens AMB8	40.00 (39.23)[f]
B. velezensis PCSE10	5.00 (11.46)[ba]
Rhizobium radiobacter PCRE10	0.00 (6.42)[a]
Streptomyces leeuwenhoekii KBT004	20.00(26.57)[ed]
B. megaterium NAT001	20.00 (26.57)[ed]
Piriformospora indica	5.00 (11.46)[ba]
Inoculated control	25.00 (29.73)[fed]
Absolute control	0.00 (6.42)[a]

*Mean of four replications having five plants each. Figures in a column followed by the same alphabet do not differ significantly according to DMRT ($P \leq 0.05$). Figures in parenthesis are arcsine transformed values.

Table 13. Incidence of bacterial wilt in the variety Naveen observed 14 days after challenge inoculation

Treatment	Percentage Disease Incidence*
Bacillus amyloliquefaciens VLY24	20.00 (26.57)[b]
B. pumilus VLY17	25.00 (29.73)[b]
Pseudomonas fluorescens PN026	40.00 (39.23)[dc]
P. fluorescens AMB8	50.00 (45.00)[d]
B. velezensis PCSE10	5.00 (11.46)[a]
Rhizobium radiobacter PCRE10	0.00 (6.42)[a]
Streptomyces leeuwenhoekii KBT004	40.00 (39.23)[dc]
B. megaterium NAT001	20.00 (26.57)[b]
Piriformospora indica	5.00 (11.46)[a]
Inoculated control	30.00 (32.90)[cb]
Absolute control	25.00 (29.73)[b]

*Mean of four replications having five plants each. Figures in a column followed by the same alphabet do not differ significantly according to DMRT (P≤ 0.05). Figures in parenthesis are arcsine transformed values.

Table 14. Incidence of bacterial wilt in the variety Naveen observed 21 days after challenge inoculation

Treatment	Percentage Disease Incidence*
Bacillus amyloliquefaciens VLY24	55.00 (47.88)[cb]
B. pumilus VLY17	65.00 (53.94)[dc]
Pseudomonas fluorescens PN026	65.00 (53.94)[dc]
P. fluorescens AMB8	70.00 (57.10)[d]
B. velezensis PCSE10	25.00 (29.73)[a]
Rhizobium radiobacter PCRE10	15.00 (21.53)[a]
Streptomyces leeuwenhoekii KBT004	60.00 (50.77)[dc]
B. megaterium NAT001	60.00 (53.94)[dc]
Piriformospora indica	60.00 (50.77)[dc]
Inoculated control	60.00 (50.77)[dc]
Absolute control	45.00 (42.12)[b]

* Mean of four replications having five plants each. Figures in a column followed by the same alphabet do not differ significantly according to DMRT ($P \leq 0.05$). Figures in parenthesis are arcsine transformed values.

Table 15. Incidence of bacterial wilt in the variety Naveen observed 7 days after challenge inoculation

Treatment	Percentage Disease Incidence*
Piriformospora indica + *Bacillus amyloliquefaciens* VLY24	0.00 (6.42)[a]
P. indica + *Streptomyces leeuwenhoekii* KBT004	0.00 (6.42)[a]
P. indica + *B. velezensis* PCSE10	5.00 (11.46)[a]
P. indica + *Rhizobium radiobacter* PCRE10	30.00 (32.90)[b]
Inoculated control	25.00 (29.73)[b]
Absolute control	0.00 (6.42)[a]

*Mean of four replications having five plants each. Figures in a column followed by the same alphabet do not differ significantly according to DMRT ($P \leq 0.05$). Figures in parenthesis are arcsine transformed values.

Table 16. Incidence of bacterial wilt in the variety Naveen observed 14 days after challenge inoculation

Treatment	Percentage Disease Incidence*
Piriformospora indica + *Bacillus amyloliquefaciens* VLY24	5.00 (11.46)[a]
P. indica + *Streptomyces leeuwenhoekii* KBT004	0.00 (6.42)[a]
P. indica + *B. velezensis* PCSE10	25.00 (29.73)[b]
P. indica + *Rhizobium radiobacter* PCRE10	55.00 (47.88)[c]
Inoculated control	30.00 (32.90)[b]
Absolute control	25.00 (29.73)[b]

* Mean of four replications having five plants each. Figures in a column followed by the same alphabet do not differ significantly according to DMRT ($P \leq 0.05$). Figures in parenthesis are arcsine transformed values.

Plants treated with combined application of *P. indica and B. amyloliquefaciens* VLY24 was successful in suppressing the disease to the highest extent when observed on the 21st day after challenge inoculation. Inoculated control showed high level of disease incidence (60.0 per cent) and was on par with plants that received treatment containing a combination of *P. indica* and *R. radiobacter* PCRE10 (Table 17).

4.7.1.3. *Root Colonization by Piriformospora indica*

Roots of all the surviving plants treated with *Piriformospora indica* and the combinations of *Piriformospora indica* with bacterial isolates were stained to assess the extent of root colonization by the fungal entophyte (Plate 11). The percentage root colonization of *P. indica* is given in Table 18. Plants treated with *Piriformospora indica* alone showed highest root colonization with 46.40 percent followed by a combination by *P. indica* with *Pseudomonas fluorescens* AMB8 (32.87 per cent), *Pseudomonas fluorescens* PN026 (30.30 percent) and *Bacillus velezensis* PCSE10 (26.77 percent).

4.7.2. *In vivo* Evaluation of Bioagents for the Suppression of Bacterial Wilt Incidence in Variety Vijay

4.7.2.2. Suppression of Bacterial Wilt Incidence by Application of Individual Bioagents

After challenge inoculation with *Ralstonia solanacearum* having a population density of 10^8cfu ml^{-1}, bacterial wilt incidence was recorded at weekly intervals for 21 days. Percentage Disease Incidence was worked out from the data and statistically analyzed.

Seven days after inoculation, no disease incidence was noticed in the plants treated with *Streptomyces leeuwenhoekii* KBT004 which was on par with the absolute control. Plants in the inoculated control exhibited highest level of disease incidence

Table 17. Incidence of bacterial wilt in the variety Naveen observed 21 days after challenge inoculation

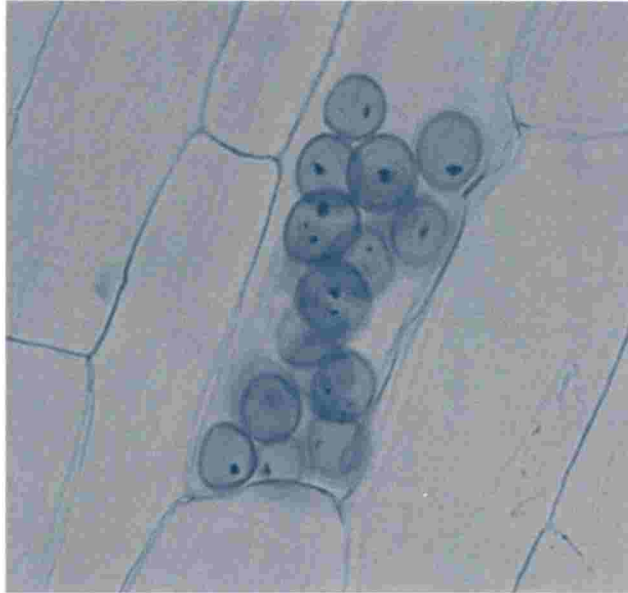
Treatment	Percentage Disease Incidence*
Piriformospora indica + *Bacillus amyloliquefaciens* VLY24	40.00 (39.23)[a]
P. indica + *Streptomyces leeuwenhoekii* KBT004	45.00 (42.12)[a]
P. indica + *B. velezensis* PCSE10	45.00 (42.12)[a]
P. indica + *Rhizobium radiobacter* PCRE10	60.00 (50.77)[b]
Inoculated control	60.00 (50.77)[b]
Absolute control	45.00 (42.12)[a]

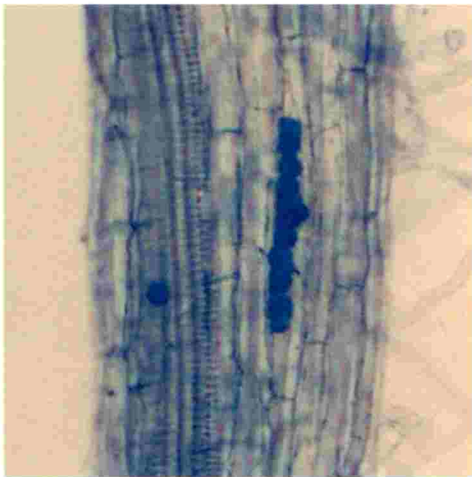
* Mean of four replications having five plants each. Figures in a column followed by the same alphabet do not differ significantly according to DMRT ($P \leq 0.05$). Figures in parenthesis are arcsine transformed values.

Table 18. Root colonization by *Piriformospora indica* in the tomato variety Naveen

Treatment	Root colonization (%) *
Piriformospora indica	46.40
Bacillus amyloliquefaciens VLY24 + *P. indica*	19.29
Rhizobium radiobacter PCRE10 + *P. indica*	24.24
B. velezensis PCSE10 + *P. indica*	26.77
Streptomyces leeuwenhoekii KBT004 + *P. indica*	23.52

* Observation from 100 root bits





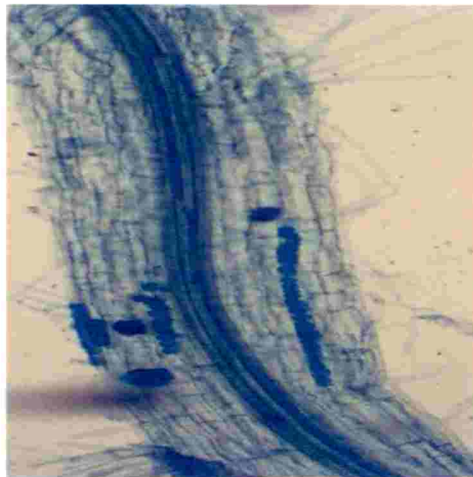


Plate 11. Colonization of *Piriformospora indica* within the root cortex cells of tomato palnts

followed by those treated with *Pseudomonas fluorescens* AMB8 and *Bacillus amyloliquefaciens* VLY24 and were statistically on par (Table 19).

On the 14th day after inoculation, the lowest disease incidence was exhibited by the plants treated with *Streptomyces leeuwenhoekii* KBT004 (5.0 percent) which was on par with absolute control which showed no disease incidence. Inoculated control showed the highest level of disease incidence (Table 20).

Minimum disease incidence was recorded in plants which was not given challenge inoculation, followed by the plants treated with *Streptomyces leeuwenhoekii* KBT004 when assessed on the 21st day after inoculation. All treatments showed significant level of disease suppression when compared to the inoculated control in which all the plant succumbed to the disease (Table 21).

4.7.2.2. Suppression of Bacterial Wilt Incidence by Combined Application of Bioagents

On the 7th day after inoculation, the lowest disease incidence was noticed in the plants grown in absolute control where no disease occurred. PDI in the combination of *Piriformospora indica* and *Rhizobium radiobacter* PCRE10 was on par with the combined treatment involving *Piriformospora indica* and *Bacillus velezensis* PCSE10 which were found to have the least disease incidence among all other treatments (Table 22).

Pathogen inoculated control showed high level of disease incidence after 14 days of challenge inoculation. The disease suppressive ability was found to be statistically similar in the treatments that involved combination of *Piriformospora indica* with *Streptomyces leeuwenhoekii* KBT004 and *Bacillus amyloliquefaciens* VLY24 (Table 23).

Table 19. Incidence of bacterial wilt in the variety Vellayani Vijay observed 7 days after challenge inoculation

Treatment	Percentage Disease Incidence*
Bacillus amyloliquefaciens VLY24	30.00 (32.90)[dc]
B. pumilus VLY17	20.00 (26.57)[c]
Pseudomonas fluorescens PN026	10.00 (16.49)[c]
P. fluorescens AMB8	30.00 (32.90)[dc]
B. velezensis PCSE10	20.00 (26.57)[c]
Rhizobium radiobacter PCRE10	10.00 (16.49)[b]
Streptomyces leeuwenhoekii KBT004	0.00 (6.42)[a]
Bacillus megaterium NAT001	20.00 (26.57)[c]
Piriformospora indica	20.00 (26.57)[c]
Inoculated control	40.00 (39.23)[d]
Absolute control	0.00 (6.42)[a]

*Mean of four replications having five plants each. Figures in a column followed by the same alphabet do not differ significantly according to DMRT ($P \leq 0.05$). Figures in parenthesis are arcsine transformed values.

Table 20. Incidence of bacterial wilt in the variety Vellayani Vijay observed 14 days after challenge inoculation

Treatment	Percentage Disease Incidence*
Bacillus amyloliquefaciens VLY24	50.00 (45.00)[c]
B. pumilus VLY17	40.00 (39.23)[cb]
Pseudomonas fluorescens PN026	30.00 (32.90)[b]
P. fluorescens AMB8	50.00 (45.00)[c]
B. velezensis PCSE10	50.00 (45.00)[c]
Rhizobium radiobacter PCRE10	40.00 (39.23)[cb]
Streptomyces leeuwenhoekii KBT004	5.00 (12.43)[a]
B. megaterium NAT001	40.00 (39.23)[cb]
Piriformospora indica	40.00 (39.23)[cb]
Inoculated control	80.00 (63.43)[d]
Absolute control	0.00 (6.42)[a]

* Mean of four replications having five plants each. Figures in a column followed by the same alphabet do not differ significantly according to DMRT ($P \leq 0.05$). Figures in parenthesis are arcsine transformed values.

Table 21. Incidence of bacterial wilt in the variety Vellayani Vijay observed 21 days after challenge inoculation

Treatment	**Percentage Disease Incidence***
Bacillus amyloliquefaciens VLY24	70.00 (57.10)[d]
B. pumilus VLY17	60.00 (50.77)[dc]
Pseudomonas fluorescens PN026	50.00 (45.00)[c]
P. fluorescens AMB8	70.00 (57.10)[d]
B. velezensis PCSE10	70.00 (57.10)[d]
Rhizobium radiobacter PCRE10	50.00 (45.00)[c]
Streptomyces leeuwenhoekii KBT004	30.00 (32.90)[b]
B. megaterium NAT001	60.00 (50.77)[dc]
Piriformospora indica	60.00 (50.77)[dc]
Inoculated control	100 (90.00)[e]
Absolute control	0.00 (6.42)[a]

* Mean of four replications having five plants each. Figures in a column followed by the same alphabet do not differ significantly according to DMRT ($P \leq 0.05$). Figures in parenthesis are arcsine transformed values.

Table 22. Incidence of bacterial wilt in the variety Vellayani Vijay observed 7 days after challenge inoculation

Treatment	**Percentage Disease Incidence***
Piriformospora indica + *Bacillus amyloliquefaciens* VLY24	30.00 (32.90)[c]
P. indica + *Streptomyces leeuwenhoekii* KBT004	30.00 (32.90)[c]
P. indica + *B. velezensis* PCSE10	20.00 (26.57)[b]
P. indica + *Rhizobium radiobacter* PCRE10	20.00 (26.57)[b]
Inoculated control	40.00 (39.23)[d]
Absolute control	0.00 (6.42)[a]

* Mean of four replications having five plants each. Figures in a column followed by the same alphabet do not differ significantly according to DMRT ($P \leq 0.05$). Figures in parenthesis are arcsine transformed values.

Table 23. Incidence of bacterial wilt in the variety Vellayani Vijay observed 14 days after challenge inoculation

Treatment	Percentage Disease Incidence*
Piriformospora indica + *Bacillus amyloliquefaciens* VLY24	60.00 (50.77)[c]
P. indica + *Streptomyces leeuwenhoekii* KBT004	70.00 (57.10)[d]
P. indica + *B. velezensis* PCSE10	50.00 (45.00)[cb]
P. indica + *Rhizobium radiobacter* PCRE10	40.00 (39.23)[b]
Inoculated control	80.00 (63.43)[e]
Absolute control	0.00 (6.42)[a]

* Mean of four replications having five plants each. Figures in a column followed by the same alphabet do not differ significantly according to DMRT ($P \leq 0.05$). Figures in parenthesis are arcsine transformed values.

On the 21st day after inoculation, 100 percent disease incidence was recorded in inoculated control which was statistically on par with the treatments that involved a combination of *Piriformospora indica* with *Bacillus amyloliquefaciens* VLY24 and *Streptomyces leeuwenhoekii* KBT004. Combined application of *Piriformospora indica* with either *Bacillus velezensis* PCSE10 or *Rhizobium radiobacter* PCRE10 suppressed the incidence of bacterial wilt disease to the tune of 50 per cent (Table 24).

4.8. PLANT GROWTH PROMOTION BY RHIZOBACTERIA, BACTERIAL ENDOPHYTE AND FUNGAL ENDOPHYTE IN VARIETY VELLAYANI VIJAY IN THE NURSERY

Plants treated with endophytic bacteria *Bacillus velezensis* PCSE10 showed the maximum plant height which was at par with those treated with a combination of *Piriformospora indica* and *Bacillus amyloliquefaciens* VLY24. Though all the treatments registered higher values with respect to plant height compared to control, there was no significant difference between the control and treatments involving single application of *Streptomyces leeuwenhoekii* KBT004, *Bacillus amyloliquefaciens* VLY24, *Bacillus megaterium* NAT001 and *Pseudomonas fluorescens* AMB8 (Table 25, Plates 12 and 13).

Plants treated with rhizobacterium, *Bacillus pumilus* VLY17 produced maximum leaf number which was on par with those treated with *Bacillus velezensis* PCSE10 and *Pseudomonas fluorescens* PN026. All other plants which were treated with bioagents either singly or in combination had lesser number of leaves than the untreated control (Table 25).

Application of *Piriformospora indica* and *Bacillus amyloliquefaciens* VLY24 in combination resulted in highest shoot fresh weight which was at par with the endophytic bacterial treatment involving *Bacillus velezensis* PCSE10. Individual

Table 24. Incidence of bacterial wilt in the variety Vellayani Vijay observed 21 days after challenge inoculation

Treatment	Percentage Disease Incidence*
Piriformospora indica + Bacillus amyloliquefaciens VLY24	90.00 (76.72)[c]
P. indica + Streptomyces leeuwenhoekii KBT004	90.00 (76.72)[c]
P. indica + B.velezensis PCSE10	50.00 (45.00)[b]
P. indica + Rhizobium radiobacter PCRE10	50.00 (45.00)[b]
Inoculated control	100.00 (90.00)[c]
Absolute control	0.00 (6.42)[a]

*Mean of four replications having five plants each. Figures in a column followed by the same alphabet do not differ significantly according to DMRT ($P \leq 0.05$). Figures in parenthesis are arcsine transformed values.

Table 25. Biometric observation of tomato variety Vellayani Vijay treated with various bioagents and their combinations

Treatment	Plant height (cm)	Leaf number / plant	Shoot fresh weight (g/plant)	Shoot dry weight (mg/plant)	Root fresh weight (g/plant)	Root dry weight (mg/plant)
Bacillus amyloliquefaciens VLY24	7.61^{gh}	5.17^{bcd}	0.60^{f}	36.97^{h}	0.11^{abcd}	6.28^{def}
Bacillus pumilus VLY17	12.38^{cde}	6.17^{a}	1.27^{cd}	77.87^{bcde}	0.09^{bcde}	12.18^{ab}
Pseudomonas fluorescens PN026	11.41^{def}	5.92^{ab}	1.09^{cdef}	56.53^{defgh}	0.09^{bcde}	8.77^{bcde}
Pseudomonas fluorescens AMB8	8.58^{fgh}	4.58^{cdef}	0.59^{f}	40.82^{gh}	0.04^{e}	3.91^{f}
Bacillus velezensis PCSE10	18.58^{a}	5.83^{ab}	1.95^{ab}	122.55^{a}	0.11^{abc}	15.04^{a}
Rhizobium radiobacter PCRE10	14.49^{bc}	4.83^{cdef}	1.41^{c}	70.84^{cdef}	0.08^{cde}	6.64^{def}
Streptomyces leeuwenhoekii KBT004	7.38^{gh}	5.25^{bc}	0.73^{ef}	36.65^{h}	0.06^{de}	4.95^{ef}
Bacillus megaterium NAT001	8.56^{fgh}	4.92^{cdef}	0.66^{ef}	45.23^{fgh}	0.12^{abc}	6.93^{def}
Piriformospora indica	13.98^{bcd}	4.83^{cdef}	1.55^{bc}	85.84^{bc}	0.12^{abc}	11.64^{abc}
P. indica+ Bacillus amyloliquefaciens VLY24	16.33^{ab}	5.00^{cde}	2.08^{a}	104.78^{ab}	0.14^{a}	10.63^{abcd}
P. indica + Streptomyces leeuwenhoekii KBT004	14.00^{bcd}	4.42^{def}	1.38^{cd}	83.14^{bcd}	0.09^{bcde}	11.39^{abc}
P.indica + Bacillus velezensis PCSE10	12.29^{cde}	4.17^{f}	1.11^{cde}	54.95^{efgh}	0.08^{bcde}	5.82^{ef}
P.indica+ Rhizobium radiobacter PCRE10	9.83^{efg}	4.25^{ef}	0.87^{def}	64.38^{cdefg}	0.13^{ab}	13.13^{ab}
Control	6.49^{h}	5.33^{bc}	0.62^{ef}	46.36^{fgh}	0.05^{e}	7.74^{cdef}

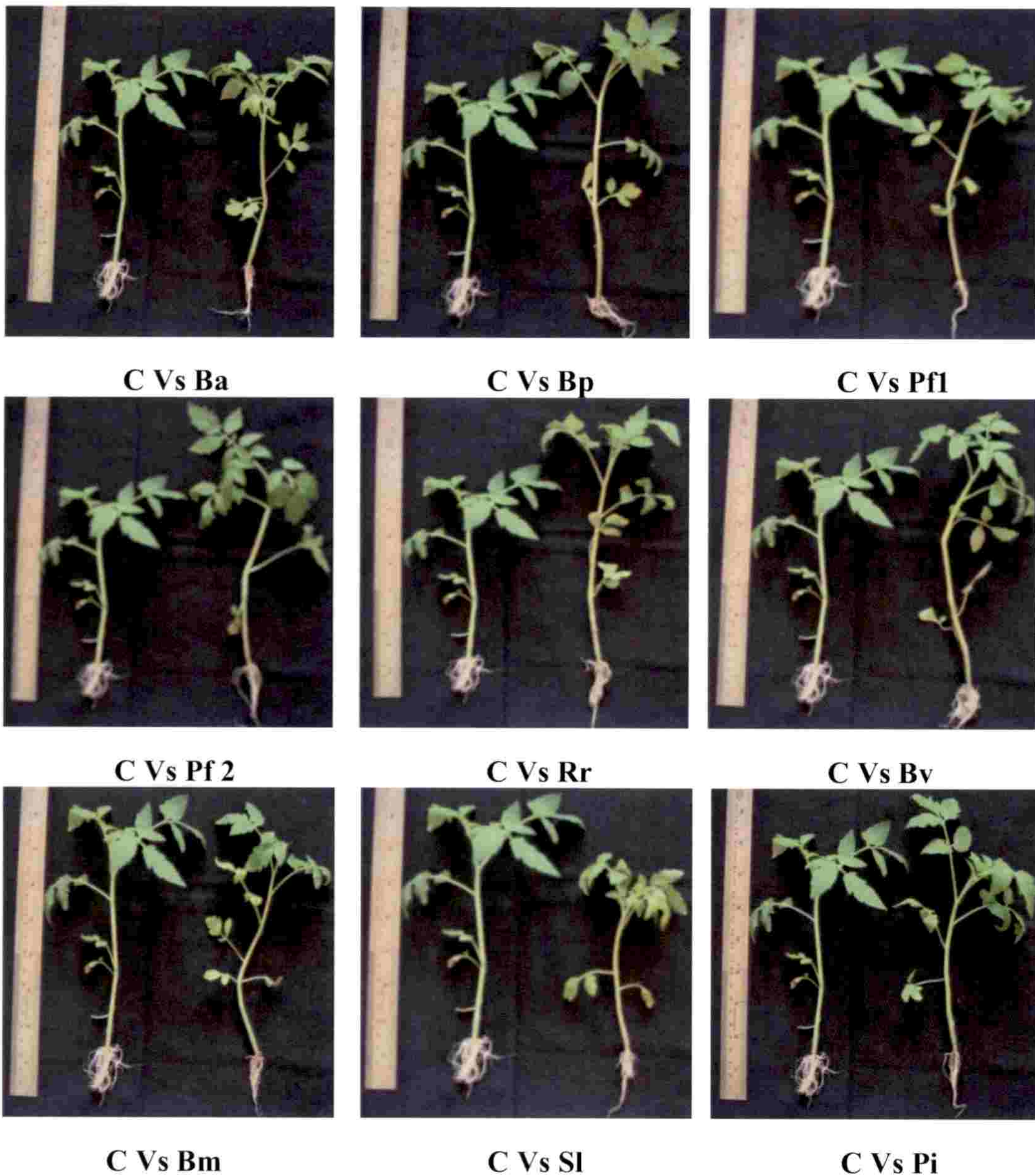
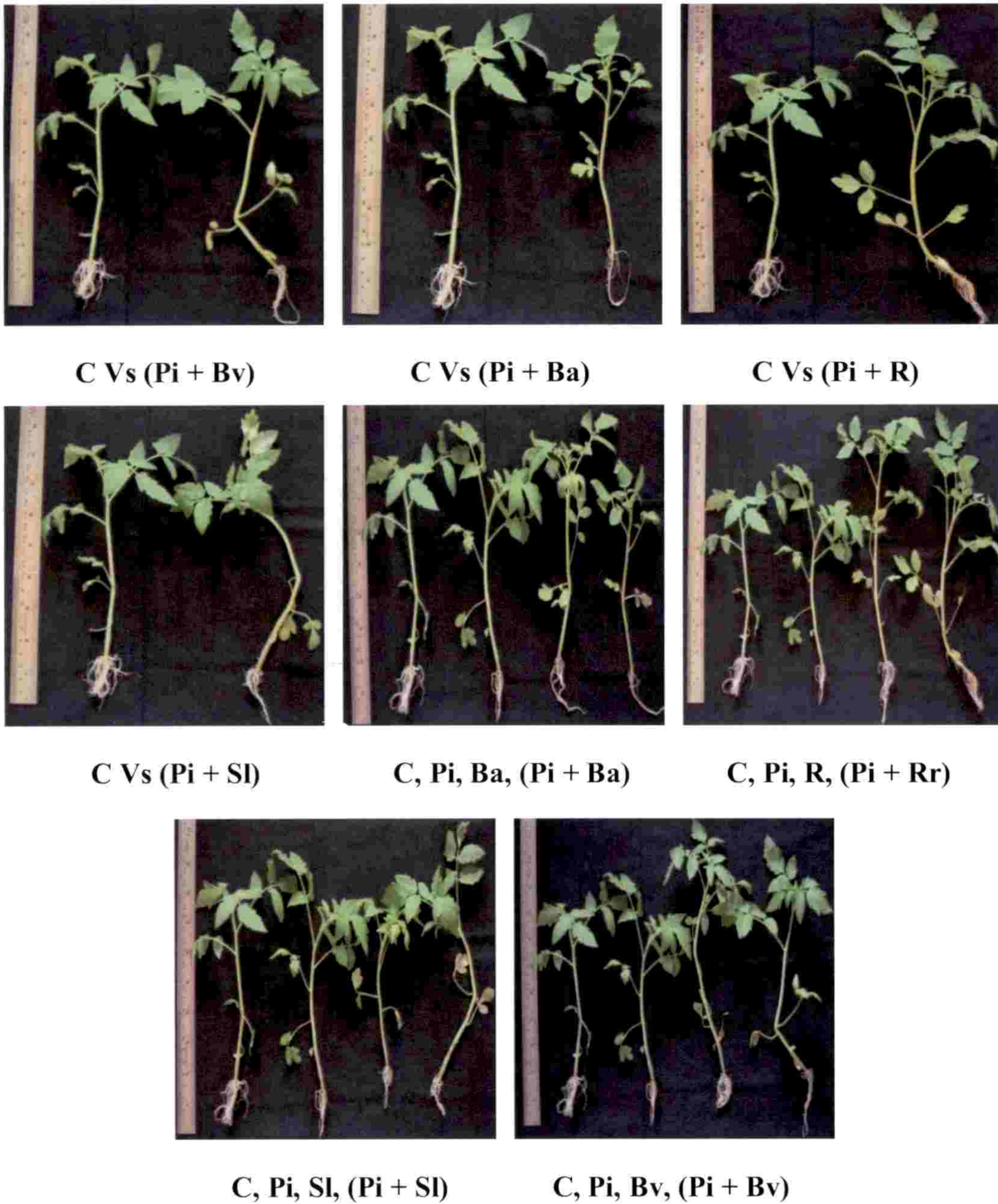


Plate 12. Assessment of plant growth promotion by bioagents in the tomato variety Vellayani Vijay

Ba- *Bacillus amyloliquefaciens* VLY24, Bp- *B. pumilus* VLY17, Pf 1- *Pseudomonas fluorescens* PN026, Pf 2- *P. fluorescens* AMB8, Rr- *Rhizobium radiobacter* PCRE10, Bv- *B. velezensis* PCSE10, Bm- *B. megaterium* NAT001, Sl- *Streptomyces leeuwenhoekii* KBT004, Pi- *Piriformospora indica*, C- Control.



C Vs (Pi + Bv)　C Vs (Pi + Ba)　C Vs (Pi + R)

C Vs (Pi + Sl)　C, Pi, Ba, (Pi + Ba)　C, Pi, R, (Pi + Rr)

C, Pi, Sl, (Pi + Sl)　C, Pi, Bv, (Pi + Bv)

Plate 13. Assessment of plant growth promotion by bioagents in the tomato variety Vellayani Vijay

Ba- *Bacillus amyloliquefaciens* VLY24, Rr- *Rhizobium radiobacter* PCRE10, Bv- *B. velezensis* PCSE10, Sl- *Streptomyces leeuwenhoekii* KBT004, Pi- *Piriformospora indica*, C- Control.

application of *Piriformospora indica*, *Rhizobium radiobacter* PCRE10, *Bacillus pumilus* VLY17 and the combination of *Piriformospora indica* and *Streptomyces leeuwenhoekii* KBT004 resulted in significantly lower fresh shoot weight than the earlier mentioned treatments (Table 25).

When shoot dry weight was analyzed statistically, significantly higher values were obtained in the case of plants that were treated with individual application of *Bacillus velezensis* PCSE10 and the combination involving *Piriformospora indica* and *Bacillus amyloliquefaciens* VLY24. Reduction in dry shoot weight was observed in individual treatments of *Bacillus amyloliquefaciens* VLY24, *Pseudomonas fluorescens* AMB8, *Streptomyces leeuwenhoekii* KBT004 and *Bacillus megaterium* NAT001compared to the untreated control. Combined application of *Piriformospora indica* with all the bacterial bioagents resulted in higher shoot dry weight than the control but it was significantly lesser from that recorded in the individual application of *Piriformospora indica* (Table 25).

Other than the individual application of *Pseudomonas fluorescens* AMB8, all other bioagents when applied singly resulted in improved root growth. The highest root fresh weight was recorded in plants that were treated with a combination of *Piriformospora indica* and *Bacillus amyloliquefaciens* VLY24 (Table 25).

Among the individual treatments, application of *Bacillus velezensis* PCSE10 registered maximum root dry weight per plant followed by *Bacillus pumilus* VLY17 and the root endophytic fungus. Treatment with combination of the endophytic fungus and bacterial bioagents except that involved *Bacillus velezensis* PCSE10, resulted in increased root dry weight than the un-inoculated control (Table 25).

Discussion

5. DISCUSSION

Ralstonia solanacearum is one of the deadly pathogen with an extremely wide host range. It causes the devastating disease, bacterial wilt, which poses a threat to solanaceous crops around the world thereby limiting their production. Direct yield losses due to the disease may vary from 33 to 90 % depending upon the biotic and abiotic factors. Complete control of this pathogen is not possible by any known control strategies till date. Using chemical control had lead to hazardous impact on the ecosystem. Biological control appears to be the best alternative to reduce the disease and minimize the risk of developing pesticide resistant strains of the pathogen. The current research focused on the usage of rhizobacteria, bacterial endophytes and the endophytic fungus *P. indica* as biocontrol agents in controlling the bacterial wilt disease in tomato.

Four rhizobacterial isolates were used in the present study and they were *B. pumilus* VLY17, *B. amyloliquefaciens* VLY24, *P. fluorescens* PN026 and *P. fluorescens* AMB8. All 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 bacterial endophytes used in the study included *R. radiobacter* PCRE10, *B. velezensis* PCSE10, *S. leeuwenhoekii* KBT004 *and B. megaterium* NAT001. *R. radiobacter* and *B. velezensis* were isolated from the exotic pepper variety *Piper colubrinum* from the stem and root respectively. They had plant growth promotional activity in black pepper plantlets and biocontrol activity against the foot rot pathogen *Phytophthora capsici* (Kollakkodan, 2017; Kollakkodan *et al.*, 2017). The strains *S. leeuwenhoekii and B. megaterium* were isolated from the roots of moderately bacterial wilt tolerant tomato variety Vellayani Vijay. These strains exhibited

enhanced seedling vigour and growth when seeds of tomato were treated with them (Vyshakhi, 2016).

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). *P. indica* has been reported in tomato as a growth promoting agent as well as a biological agent for disease suppression (Fakhro *et al.*, 2010; Varkey *et al.*, 2018)

The whole course of the research was divided into two, *in vitro* and *in vivo* experiments. As a preliminary step to initiate the study, isolation of bacterial wilt pathogen from the stem of the diseased plants showing typical bacterial wilt symptoms was performed. The symptoms of infected plants in the field included sudden wilting of younger foliage, stunting of the plants, vascular discoloration and formation of adventitious roots in the collar region as described elsewhere (Gota, 1992). The occurrence of the disease in the suspected plant was confirmed by the preliminary test for bacterial ooze also known as streaming test, in which a white thread like dense oozing would be observed when the cut end of the stem of the plant was kept in physical contact with the water. The ooze is due to the excretion of bacterial cells from the cut end of the stem to the water which gives the physical evidence of the disease severity (Champoiseau *et al.*, 2009; Chakravarthy and Kalita, 2012). The pathogen was isolated on to SMSA medium, which is a selective medium for isolating *R. solanacearum*. The selectivity is due the presence of antibiotic supplements tyrothricin, cychoheximide, bacitracin, captan, vancomycin, chloramycetin and penicillin G that prevents the growth of almost all other bacterial

species. TTC solution is also added to the medium to help detect the bacterial growth. The colonies appear fluidal with creamy-white in color with red centre on SMSA after 48h of incubation at 28°C (Pradhanang *et al.*, 2000). The nature and appearance isolates obtained in the present procedure also were in conformity with the earlier reports.

The pathogen obtained was further tested for its pathogenicity by performing Koch's postulates by inoculating healthy tomato plants with the causative organism and re-isolating the same on SMSA medium (Hong *et al.*, 2011). It was proved that the isolated pathogen from the suspected plant was the causative agent and the isolated pathogen was virulent enough to induce the disease. Further the pathogen was grown in CPG broth, from which the bacterial growth dynamics was evaluated by direct and indirect mechanism by visible plate count and spectrophotometric measurements respectively (Priou *et al.*, 2006) which helped in deriving the inoculum density (Bertolla *et al.*, 1997). Spectrophotometric measurement of the turbidity of the suspension of *R. solanacearum* growth in the CPG medium has been used for determining the cell density of the inoculum during challenge inoculation (Anith *et al.*, 2004).

As a first step to understand the microbial interaction between the pathogen and the bioagents, *in vitro* evaluation of antagonism of bioagents against the pathogen was performed. *In vitro* trials are preliminary screening methods, which can be used to narrow down to putative efficient strains (Anith *et al.*, 2003; Lemessa and Zeller, 2007). This would help to obtain the most effective strains 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 mediate the suppression of the pathogen.

Direct antagonism of a bacterial pathogen by other bacterial isolates can be checked by different methods *viz.* cross streak plate assay, agar plug diffusion technique, disc diffusion method and spot on lawn method (Balouiri *et al.*, 2016). Eight bacterial bioagents namely, *B. pumilus* VLY17, *B. amyloliquefaciens* VLY24, *P. fluorescens* PN026, *P. fluorescens* AMB8, *R. radiobacter* PCRE10, *B. velezensis* PCSE10, *S. leeuwenhoekii* KBT004 *and B. megaterium* NAT001 were tested against the bacterial wilt pathogen. In the experiment, *B. amyloliquefaciens* VLY24, *B. velezensis* PCSE10 and *S. leeuwenhoekii* KBT004 were found to have antagonism against the pathogen (Table 26).

In all the techniques mentioned above, though the results obtained were consistent, variations were observed in the inhibition zones. Inhibition zone was found to be the greatest in cross streak assay as compared to other methods. The reason for this could be attributed to the fact that the bioagent is allowed to establish in the agar medium well before the pathogen is inoculated by providing an incubation period of 48 hours. The bioagents might have secreted their secondary metabolites or any other compounds which might have induced growth inhibition of the pathogen effectively. The size of inhibition zone in each of the remaining methods exhibited a decreasing trend in the order in agar plug diffusion technique, spot on lawn method and disc diffusion method. In agar plug diffusion technique, direct contact of the pathogen with the bioagent may be the reason for the larger inhibition zone. Lesser inoculum used in disk diffusion method as compared to that of spot on lawn method, may be the reason for its comparatively reduced inhibition. Twenty μl bioagent was directly placed on the pathogen seeded medium in the spot on lawn method, unlike in the disk diffusion method in which 10 μl of the culture was inoculated on filter paper which was then placed on the plate containing the pathogen. Thus, the lesser

Table 26. Comparison of *in vitro* antagonism by bioagents against *Ralstonia solanacearum*

	Methods of testing antagonism					
	Direct			Indirect		
Organism	CSPA	APDA	DDM	SOLM	AWA	DDA
Bacillus amyloliquefaciens VLY24	+	+	+	+	+	+
B. pumilus VLY17	-	-	-	-	-	-
Pseudomonas fluoresens PN026	-	-	-	-	-	-
P. fluoresens AMB8	-	-	-	-	-	-
Rhizobium radiobacter PCRE10	-	-	-	-	-	-
B. velezensis PCSE10	+	+	+	+	+	+
B. megaterium NAT001	-	-	-	-	-	-
Streptomyces leeuwenhoekii KBT004	+	+	+	+	-	-
Piriformospora indica	ND	ND	ND	ND	+	+

CSAP- Cross streak plate assay; APDA- Agar plug diffusion assay; DDM- Disc diffusion method; SOLM- Spot on lawn method; AWA- Agar well assay; DDA- Disc diffusion assay; ND- Not done

inoculum concentration in the latter method may be the reason for reduced inhibition zone.

In vitro tests as mentioned above have been used for screening for antagonism against the bacterial wilt pathogen earlier. Four species of *Bacillus* including *B. subtilis and B. pumulis* and *P. fluorecsens* were found to have strong antagonism against *R. solanacearum* in cross streak inhibition assay (Aliye *et al.*, 2008). Similar screening was done by Hu *et al.* (2010) for checking the antagonism of *B. amyloliquefaciens* against capsicum bacterial wilt pathogen by agar disk diffusion method. Antagonism of *B. velezensis* against *R. solanacearum* by spot on lawn method was reported by Cao *et al* (2018). Boukaew *et al.* (2011) reported the antagonism of *S. leeuwenhoekii* against the bacterial wilt pathogen in agar well diffusion inhibition assay. Ramesh *et al.* (2009) reported the antagonistic effect of different biovars of *P. fluorescens* against *R. solanacearum* by carrying out agar well diffusion inhibition assay. Many strains of *P. fluorescens* were found to be effective in producing siderophores which shows antibiosis against the pathogen and improves the plant health.

B. pumilus a gram-positive, aerobic, spore-forming bacteria found abundant in soil rhizosphere was used here since it has the ability to reduce the wilt incidence and help in promoting the growth of the plants to a greater extent (Anith *et al.*, 2004). Shen *et al.* (2018) tested the antagonism of *B. pumulis* WP8 against tomato bacterial wilt by dual culture method and inhibition zone was obtained. However, the strain *B. pumilus* VLY17 failed to exhibit any inhibition and this could be attributed to strain to strain difference.

Indirect method to test for antagonism was done by using cell free extract in which the culture filtrate obtained from the broth culture was used for analyzing inhibitory effect on the pathogen. Cell free extract acts as an enzymatic system devoid of cellular barriers usually having faster reaction rate than the microbial

system. The sub-cellular fraction is composed of water soluble metabolites and enzymes. In the present study, cell free extracts of bacterial bioagents and the fungal bioagent were tested for antagonism against the pathogen by two techniques namely, agar well diffusion method and disk diffusion method (Table 26).

Culture filtrate of 109 isolates of endophytic bacteria from eggplant, 23 isolates from cucumber and 12 isolates from groundnut were tested for antagonism against bacterial wilt pathogen in eggplant by agar diffusion assay and results showed that 34 isolates from eggplant, 14 isolates from cucumber and 4 isolates from groundnut tend to inhibit the growth of the pathogen (Ramesh *et al.*, 2009). Cell free culture filtrate of about twenty two isolates of antagonists including rhizobacteria and endophytic bacteria constituting different species of *Pseudomonas* and *Bacillus* were found to be effectively inhibiting *R. solanacearum* isolated from wilted eggplant in a study conducted by Ramesh and Phadke in 2012. The results from the present assay showed that culture filtrates of *B. amyloliquefaciens*VLY24, *B. velezensis* PCSE10 and *P. indica* exhibited antagonism against the pathogen. Of which *B. velezensis* PCSE10 tend to have greater inhibition than *B. amyloliquefaciens*VLY24. The zone of inhibition for *B. velezensis* PCSE10 was more with the cell free extract than with the whole culture itself. This may be due to increased amount of secreted metabolites into the broth culture during the growth phase. Besides, *S. leeuwenhoekii* KBT004, which exhibited antagonism in direct assays did not inhibit the pathogen when culture filtrate was used which may be due to inability of the organism to secrete the metabolites into the broth culture. Boukaew *et al.* in 2011 obtained fourteen isolates of *Streptomyces* spp. and tested the same for their ability to suppress bacterial wilt pathogen by the agar well diffusion inhibition assay using culture filtrate and three strains were reported to have effective inhibition on the pathogen.

B. amyloliquefaciens D29, *B. amyloliquefaciens* Am1, *B. subtilis* D16 and *B. methylotrophicus* H8 were tested for antagonism against bacterial wilt pathogen of tomato by agar well diffusion assay. The culture filtrate of all the four *Bacillus* strains

along with chitosan (5 mg/ml) resulted in inhibition of *Ralstonia* growth in comparison to corresponding control (Almoneafy *et al.*, 2014). Maji and Chakrabartty in 2014 used culture filtrates of *Pseudomonas* spp. which exhibited zone of growth inhibition on *R. solanacearum* Tom5.

Understanding the compatibility among the individual microbial components is a major prerequisite for development of a microbial consortium or mixture. *In vitro* testing for the presence or absence of antagonism between the component microbials can throw light on the nature of possible interaction among them (Nair and Anith, 2009; Varkey *et al.*, 2018). Dual culture plate assay on an agar medium that could support the growth of both the bioagents would reveal possible antagonism by exhibiting inhibition zones of growth. Though in the real field condition this relationship may not get reflected in its actual potential, this assay can be considered as a preliminary screening procedure for making out microbial interactions.

Dual culture plate assay was done to test the compatibility of bacterial bioagents with *P. indica* on PDA as both the fungal endophyte and the bacterial agents could grow well on it. Compatibility is assessed by lack of any inhibition zone whereas, the non compatible ones would develop zone of inhibition. In the present screening, *R. radiobacter* PCRE10, *S. leeuwenhoekii* KBT004 and *B. megaterium* NAT001 were found to be compatible with *P. indica*. *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*. However, 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 which was similar to that of the result obtained in the present study using the same strains. Thus, it can be concluded that the two strains mentioned above are incompatible with the endophytic fungus. In the former case however the screening was done on a different medium than PDA. This indicates the influence of the screening medium in determining the interaction between the microorganisms or variability among the isolates.

Compatible bioagents were selected for co-culture based on the results obtained in the dual culture assay. 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.

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 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. 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). In the current study, co-culture experiment was carried out using *R. radiobacter* PCRE10, *S. leeuwenhoekii* KBT004 and *B. megaterium* NAT001 with *P. indica* in potato dextrose broth. The population of inoculated bacteria was assessed to find whether the bioagents are compatible to be grown together. *B. megaterium* NAT001 failed to grow along with the fungal bioagent which may be due to the inefficiency of the bacterium to use the left over media in which *P. indica* was grown.

Table 27. Comparison of bacterial wilt incidence (%) in the tomato varieties Naveen and Vellayani Vijay on inoculation with bioagents individually

Treatment	Days after inoculation					
	7th day		14th day		21st day	
	Naveen	**Vijay**	**Naveen**	**Vijay**	**Naveen**	**Vijay**
Bacillus amyloliquefaciens VLY24	10	30	20	50	55	70
B. pumilus VLY17	15	20	25	40	65	60
Pseudomonas fluorescens PN026	30	10	40	30	65	50
P. fluorescens AMB8	40	30	50	50	70	70
B. velezensis PCSE10	5	20	5	50	25	70
Rhizobium radiobacter PCRE10	0	10	0	40	15	50
Streptomyces leeuwenhoekii KBT004	20	0	40	5	60	30
B. megaterium NAT001	20	20	20	40	60	60
Piriformospora indica	5	20	5	40	60	60
Inoculated control	25	40	30	80	60	100
Absolute control	0	0	25	0	45	0

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. In the nursery sterile soil system was used as it would encourage better colonization by the applied bioagents as completion 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.

One of the factors that influence biological control by applied antagonists is the innate level of tolerance of the crop varieties to the pathogen attack. The degree of tolerance to the pathogen can supplement the biocontrol potential (Cortesero *et al.*, 2000). In tomato also incidence of bacterial wilt in biocontrol experiments has been influenced by the crop variety (Anith *et al.*, 2004).

In the current study, two varieties of tomato, Naveen and Vellayani Vijay were used to evaluate the efficiency of eight bacterial bioagents and the fungal endophyte *P. indica,* each individually and in combination with the fungus in suppressing the bacterial wilt incidence. The variety Naveen is a high yielding hybrid variety released by Indo American Hybrid Seeds, Banguluru with a very high susceptibility to the bacterial wilt disease. Vellayani Vijay is reported to be having moderate resistance to the bacterial wilt under field conditions.

The microbial combinations for the evaluation were worked out based on the results obtained during the *in vitro* tests. *B. amyloliquefaciens* VLY24, *S. leeuwenhoekii* KBT004 and *B. velezensis* PCSE10 showed antagonism against the bacterial pathogen in the *in vitro* assays. In the compatibility assay of the antagonists with the root endophytic fungus, it was observed that *S. leeuwenhoekii* KBT004, *R. radiobacter* PCRE10 and *B. megaterium* NAT001 were compatible with *P. indica.* However *B. megaterium* NAT001 failed to grow in the co-culture system. Based on

the antagonistic property, compatibility and the ability to be co-cultured with the endophytic fungus, selection of the bacterial agents for mixed inoculation with *P. indica* was done. Therefore *B. amyloliquefaciens* VLY24, *S. leeuwenhoekii* KBT004, *B. velezensis* PCSE10 and *R. radiobacter* PCRE10 were selected for combinational treatments in the pot culture experiments.

Bacterial wilt incidence during the experiments, both with the individual and combined treatments of the bioagents, differed with respect to the variety of tomato used (Table 27; Fig 3 to 6). Contradictory to the claims of being a field tolerant variety, Vellayani Vijay showed 100 % mortality in the inoculated control after 21 days of challenge inoculation whereas in the variety Naveen, mortality was 60 % only. However in the uninoculated control no plants succumbed to death in the case of Vellayani Vijay whereas the mortality was 45 % in the case of Naveen. The pathogen that could be present in the unsterile soil used for the study may have caused the disease. The population level of *R. solanacerum* present in the soil is an important factor responsible for the initiation of bacterial wilt disease (Michel and Mew, 1998; van Elsas *et al.*, 2000). It is inferred that the population that was present in the soil was able to induce disease in the highly susceptible variety but not in the moderately tolerant variety. Plant genetic factors play an important role in multiplication of the bacterial wilt pathogen within the tissues and only when a threshold population of the pathogen is reached in the plant tissues, wilt symptoms do occur. The native bacterial population in soil may have initiated the colonization within the root tissues in both the cases, but Naveen being a highly susceptible variety, the pathogen might have multiplied in a faster rate and got established in the plant system. This might have resulted in the occurrence of wilt symptoms in Naveen. However, in the case of Vellayani Vijay, though the pathogen might have colonized, the innate plant factors might not have permitted its multiplication thereof, thereby not expressing the disease symptoms. In cases of many host plants, including weeds, there would be presence of the bacterial wilt pathogen in the plant tissues without

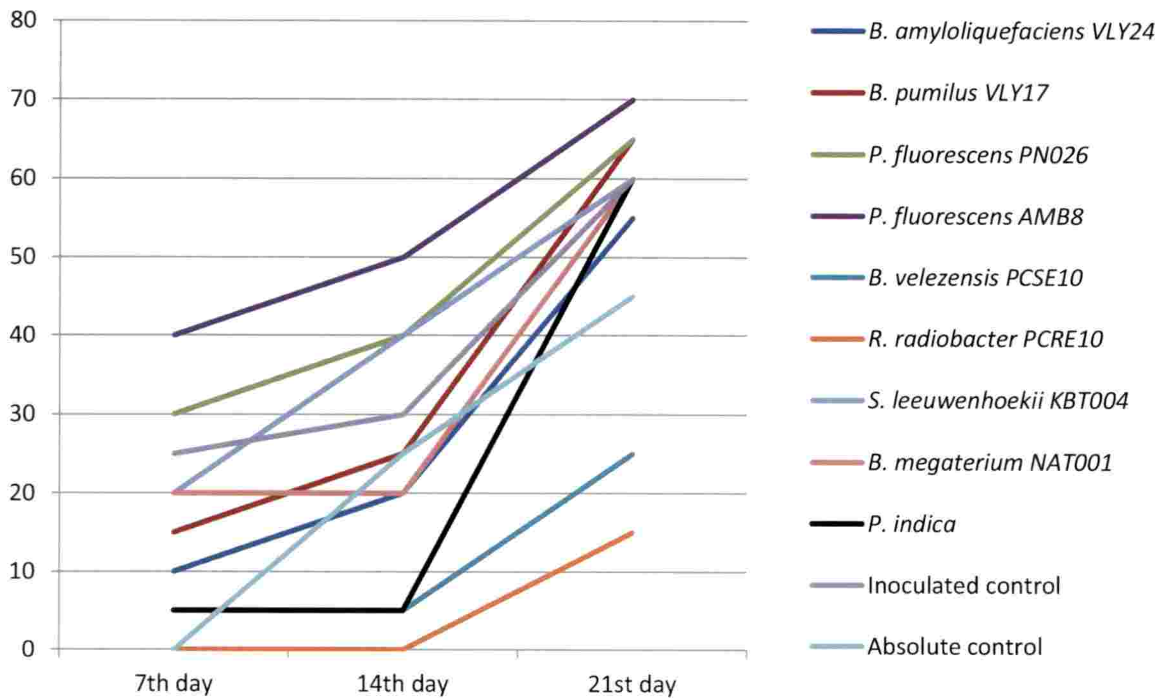


Figure 3. Disease progression in the variety Naveen treated with individual bioagents after challenge inoculation

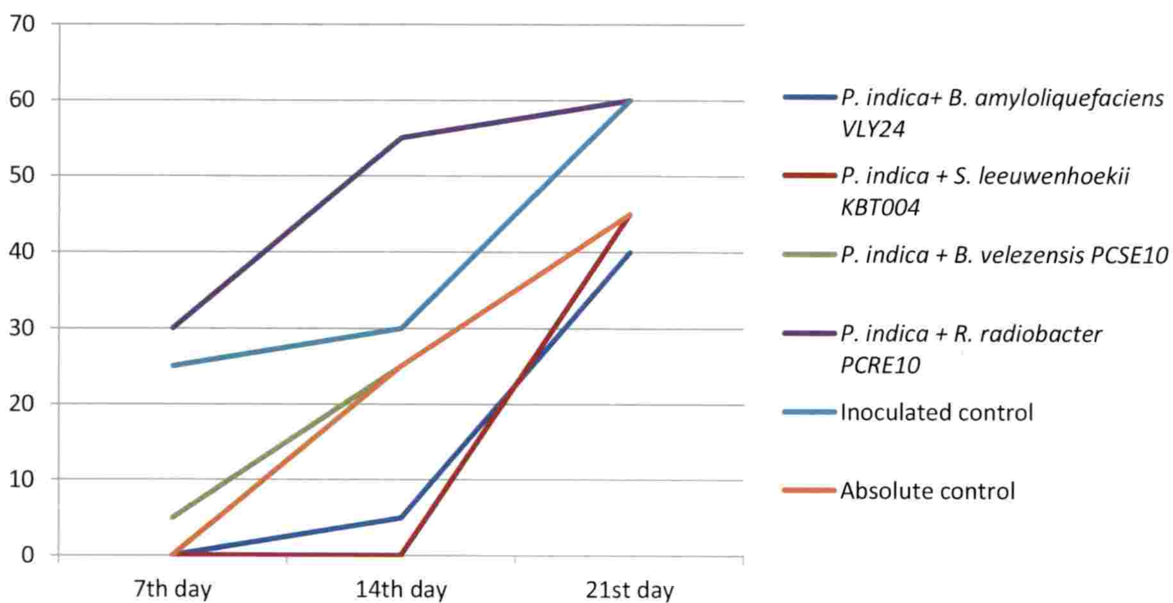


Figure 4. Disease progression in the variety Naveen treated with combination of bioagents after challenge inoculation

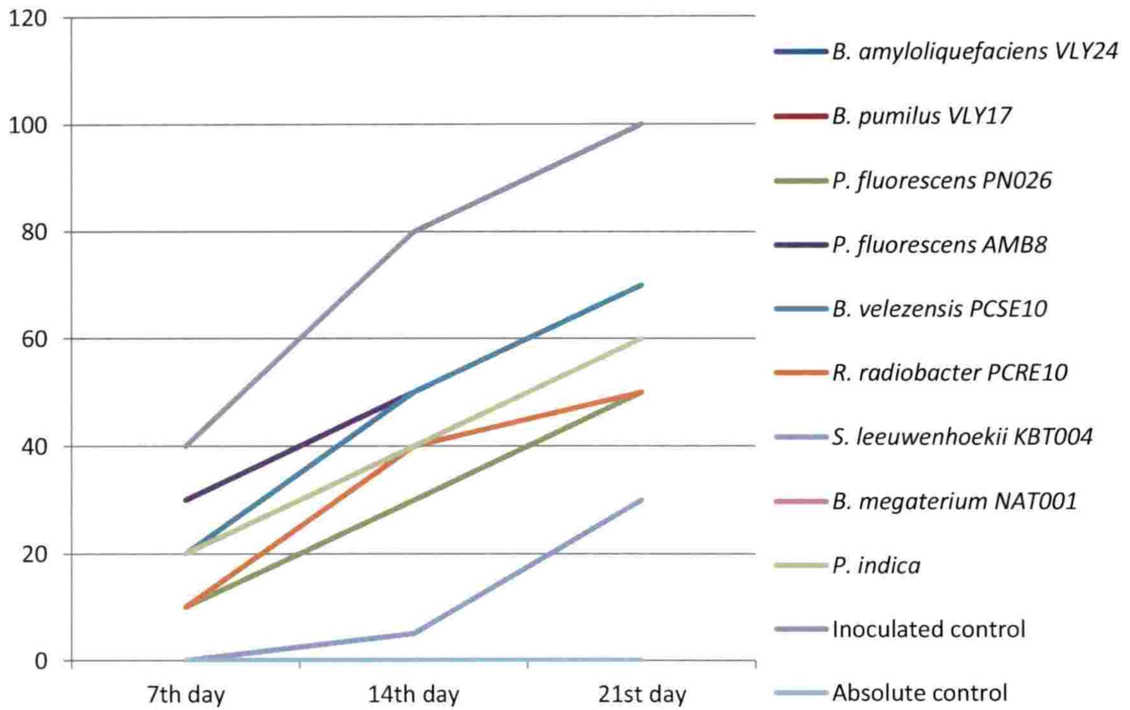


Figure 5. Disease progression in the variety Vellayani Vijay treated with individual bioagents after challenge inoculation

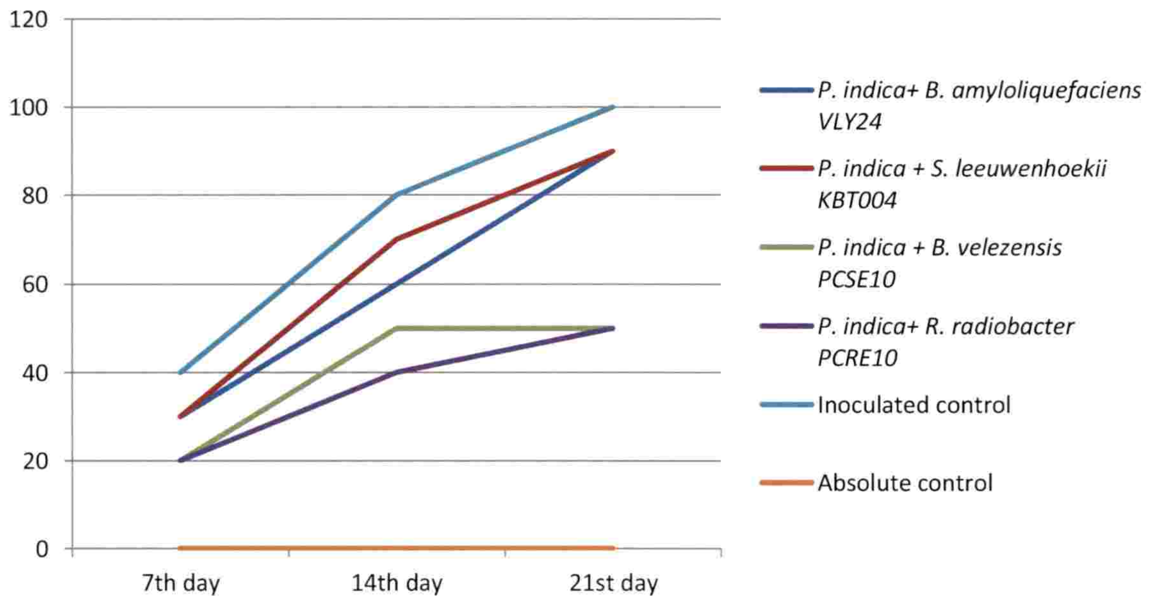


Figure 6. Disease progression in the variety Vellayani Vijay treated with combination of bioagents after challenge inoculation

getting the symptoms expressed. A colonization assay with the stem tissues in a highly specific bacteriological medium like SMSA would have given the precise answer to this question (Tusiime *et al.*, 1998; Pradhanang *et al.*, 2000; Dittapongpitch and Surat, 2003).

When the disease progression in Naveen was analyzed, it was found that seeds treated with *R. radiobacter* PCRE10 showed the minimum expression of symptoms over a period of 21 days (Fig 7 to 9). The percentage disease incidence was only 15 % as compared to 60 % in the control plants. There was also no occurrence of the disease symptoms till two weeks after challenge inoculation. *R. radiobacter* PCRE10 is an endophytic bacterial strain isolated from the wild pepper *Piper colubrinum.* It has been reported that the isolate exhibits *in vitro* antifungal activity against the foot rot pathogen, *Phytophthora capsici.* It also was able to suppress the foliar lesion development in black pepper plants under *in vivo* conditions (Kollakkodan, 2017; Kollakkodan *et al.*, 2017). However in the variety Vellayani Vijay the percentage disease incidence was found to be 50 % when the same strain has been used for seed treatment. Differential efficacy of bacterial strains with respect to root colonization and biocontrol efficiency has been reported when the same strain is used in different varieties of the same crop plant (Anith *et al.*, 2004) Another endophytic bacterium, *B. velezensis* PCSE10, that was obtained from the same wild pepper plant which also showed *in vitro* inhibition of the pathogen was also able to suppress the wilt incidence to a significant level (25 %) in the variety Naveen. Two *B. velezensis* strains that produced lipopeptide (LP) antibiotics iturin and fengycin have been reported to be efficient biocontrol agents against *R. solanacearum* (Cao *et al.*, 2018). They have observed inhibition of the growth of the bacterial wilt pathogen under *in vitro* conditions with methanolic extract of as well the whole cells. It has been observed in the present study that *B. velezensis* PCSE10 also produced inhibition when whole cells or the culture filtrates were tested against the wilt pathogen. Incidentally, this strain too was unable to help Vellayani Vijay to combat the disease

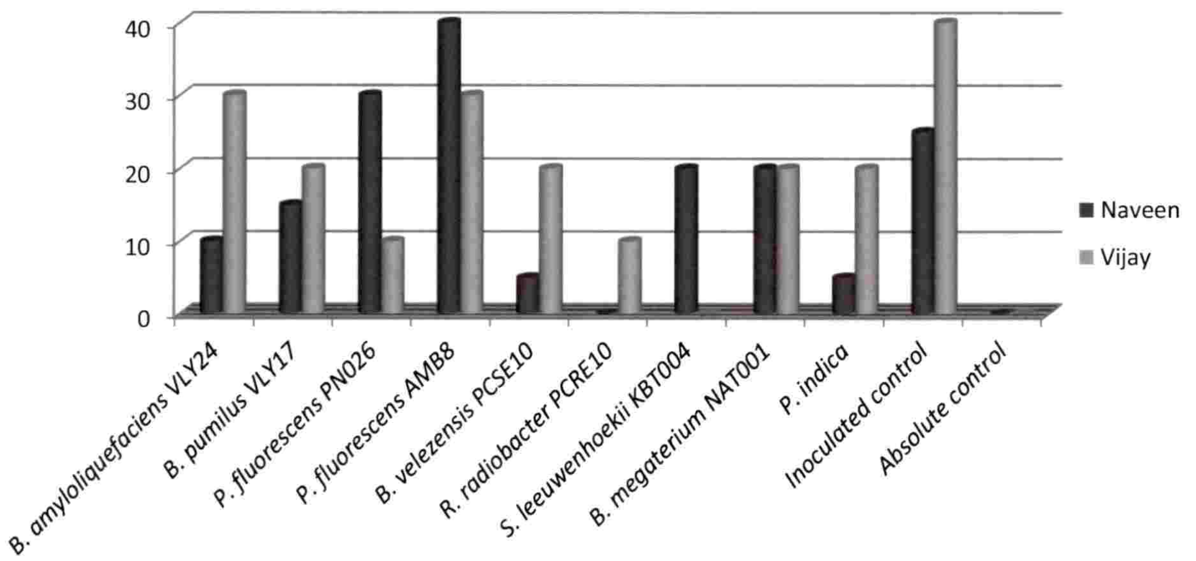


Figure 7. Comparison of disease incidence in the tomato varieties Naveen and Vellayani Vijay treated with individual bioagents after 7th day of challenge inoculation

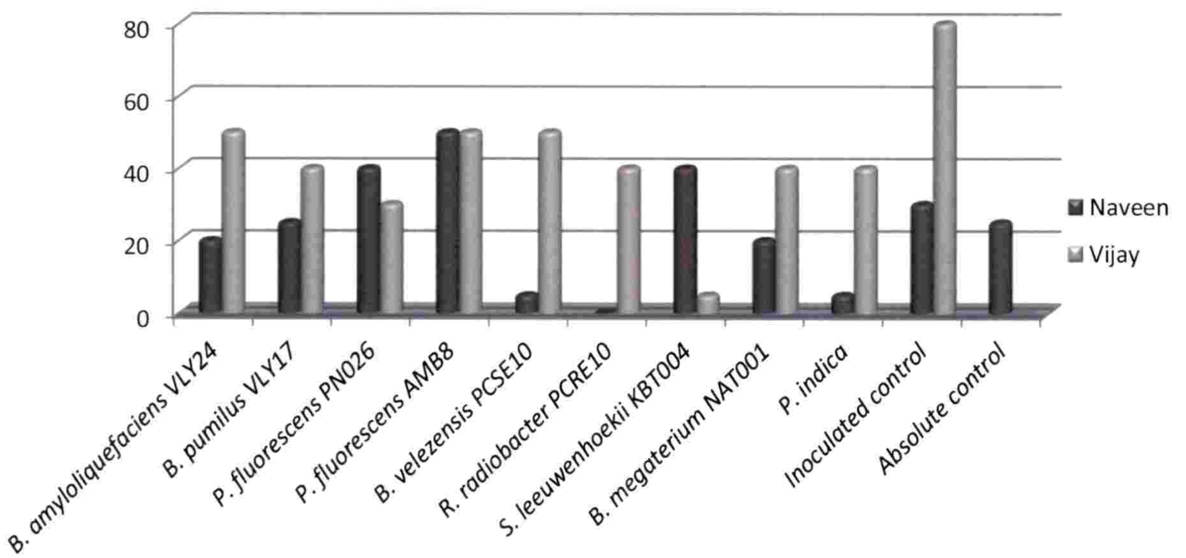


Figure 8. Comparison of disease incidence in the tomato varieties Naveen and Vellayani Vijay treated with individual bioagents after 14th day of challenge inoculation

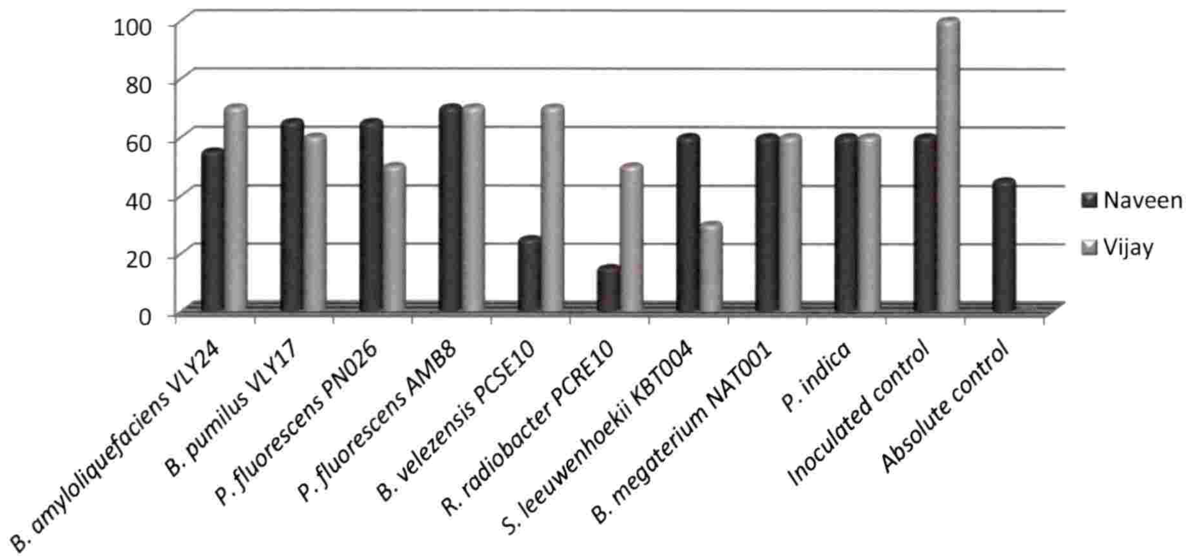


Figure 9. Comparison of disease incidence in the tomato varieties Naveen and Vellayani Vijay treated with individual bioagents after 21st day of challenge inoculation

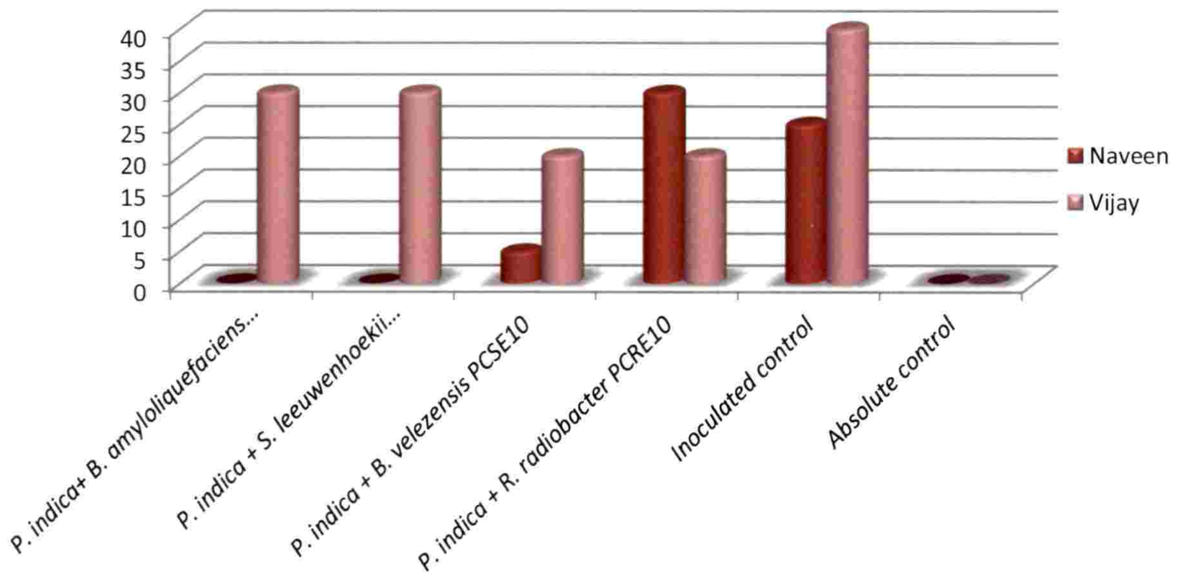


Figure 10. Comparison of disease incidence in the tomato varieties Naveen and Vellayani Vijay treated with combination of bioagents after 7th day of challenge inoculation

as in the case of *R. radiobacter* PCRE10. The percentage disease incidence was as high as 70%. It is interesting to note that both the endophytic bacteria obtained from the wild pepper species interacted positively with the variety Naveen but did not have the same effect with the variety Vellayani Vijay.

The best individual treatment in the case of Vellayani Vijay was seed treatment with *S. leeuwenhoekii* KBT004 with respect to the bacterial wilt incidence (Fig 7 to 9). It is an endophytic bacterial isolate obtained from the tomato variety Vellayani Vijay itself in a previous study (Vyshakhi, 2016). Therefore it may have a preference for the variety as a natural host. Further, it was also observed that *S. leeuwenhoekii* KBT004 had *in vitro* inhibition against the wilt pathogen. Many of the resistant varieties of tomato have been reported to have increased presence of antagonists within them as endophytes (Upreti and Thomas, 2015). The presence of antagonistic endophytes in the host plant is thought to supplement the resistance against pathogenic microorganisms. Bacterization with *B. megaterium* NAT001, though was also an endophyte derived from the tomato variety Vellayani Vijay, did not have differential influence on the two varieties tested. In both the varieties, 60 % wilt incidence has been recorded after 21 days. However, in the case of Vellayani Vijay there was a reduction of 40 % wilt incidence compared to the pathogen inoculated control when the strain was used (Fig 13). The isolate was unable to produce *in vitro* inhibition against the bacterial wilt pathogen. This shows the need for a preliminary screening through *in vitro* assays before selecting bio agents for biological control experiments.

Though the versatile bacterium, *P. fluorescens* has been implicated in biological control of several soil borne disease including bacterial wilt (Ramesh et al., 2009; Zhou *et al.*, 2012), both isolates of *P. fluorescens* tried in the current experiment failed to produce positive results. In previous studies both of the strains have been found to improve growth in several crop plants including tomato (Anith *et al.*, 2003; 2004; Anith, 2009). Both the strains have been able to suppress foot rot

disease induced by *Phytophthora capsici* in black pepper also. Anti-fungal property of a strain may not therefore be always broad spectrum antimicrobial in nature as the nature and mechanism of inhibition differs.

The axenically cultivable root endophytic fungus *P. indica*, interacts with Several plant species and promotes their growth (Weiss *et al.*, 2004; Oelmüller *et al.*, 2009; Varma *et al.*, 2012). It also enhances plant resistance to biotic stresses such as fungal, viral and nematode diseases and abiotic stresses like heavy metals, salinity and drought (Deshmukh and Kogel, 2007; Sherameti *et al.*, 2008; Daneshkhah *et al.*, 2013, Lakshmipriya, 2016; Li et al., 2017; Varkey *et al.*, 2018). Both the tomato varieties used in the present study showed similar reaction when the root endophyte was used as a biological control agent against the bacterial wilt pathogen. Though the wilt incidence was the minimum in the variety Naveen during the initial two weeks (5 %) it progressed to 60 % after 21 days of inoculation. The final wilt incidence in the case of Vellayani Vijay was also 60 %. This is for the first time that *P. indica* is reported to be a biological control agent against bacterial wilt disease caused by *R. solanacearum* (Table 28; Fig 10 to 12).

The concept of combined inoculation of bioagents in biocontrol programmes and plant growth promotion experiments arise out of the assumption that mixed inoculation or a consortium always would have added advantage over the individual application (Janisiewicz and Bors, 1995; Raupach and Kloepper, 1998; Jetiyanon and Kloepper, 2002). Various experiments have validated the concept in many crop-pathogen interactions. In the current study the combinations were worked out based on two criteria. Compatibility between the fungal endophyte and the bioagents and *in vitro* inhibition of the bioagents against the bacterial wilt pathogen. *B. amyloliquefaciens* VLY24, *S. leeuwenhoekii* KBT004 and *B. velezensis* PCSE10 possessed antagonism against the bacterial pathogen in the *in vitro* assays (Table 26). In the compatibility assay with the root endophytic fungus, it was noticed that *S. leeuwenhoekii* KBT004, *R. radiobacter* PCRE10 and *B. megaterium* NAT001 were

Table 28. Comparison of bacterial wilt incidence (%) in the tomato varieties Naveen and Vellayani Vijay on inoculation with bioagents in combination with *Piriformospora indica*

Treatment	**Days after inoculation**					
	7th day		**14th day**		**21st day**	
	Naveen	**Vijay**	**Naveen**	**Vijay**	**Naveen**	**Vijay**
Piriformospora indica+ *Bacillus amyloliquefaciens* VLY24	0	30	5	60	40	90
P. indica + *Streptomyces leeuwenhoekii* KBT004	0	30	0	70	45	90
P. indica + *B. velezensis* PCSE10	5	20	25	50	45	50
P. indica + *Rhizobium radiobacter* PCRE10	30	20	55	40	60	50
Inoculated control	25	40	30	80	60	100
Absolute control	0	0	25	0	45	0

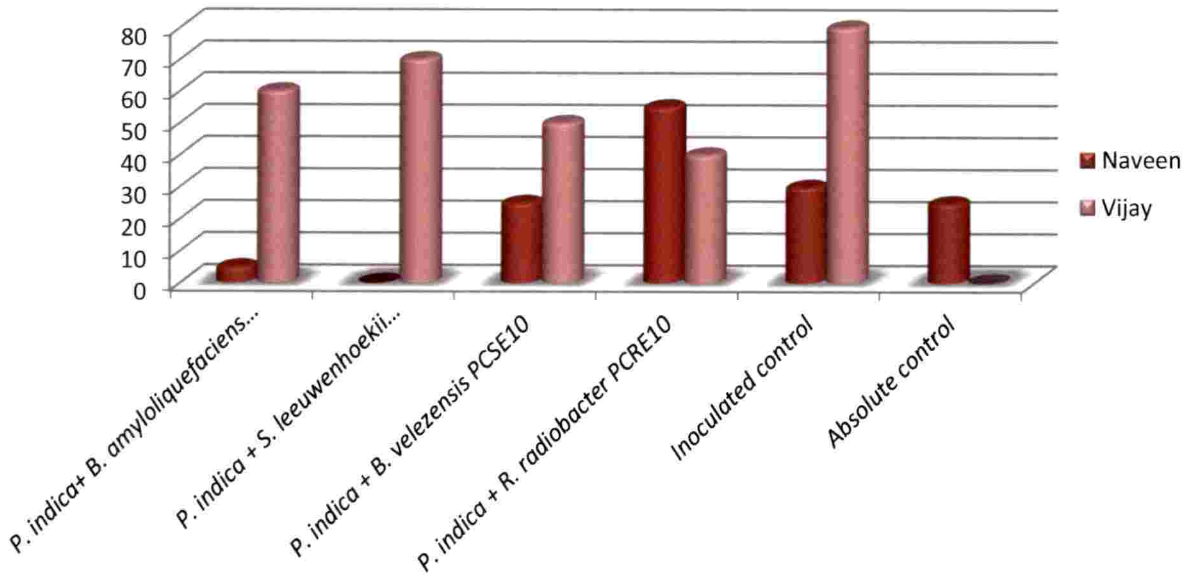


Figure 11. Comparison of disease incidence in the tomato varieties Naveen and Vellayani Vijay treated with combination of bioagents after 14th day of inoculation

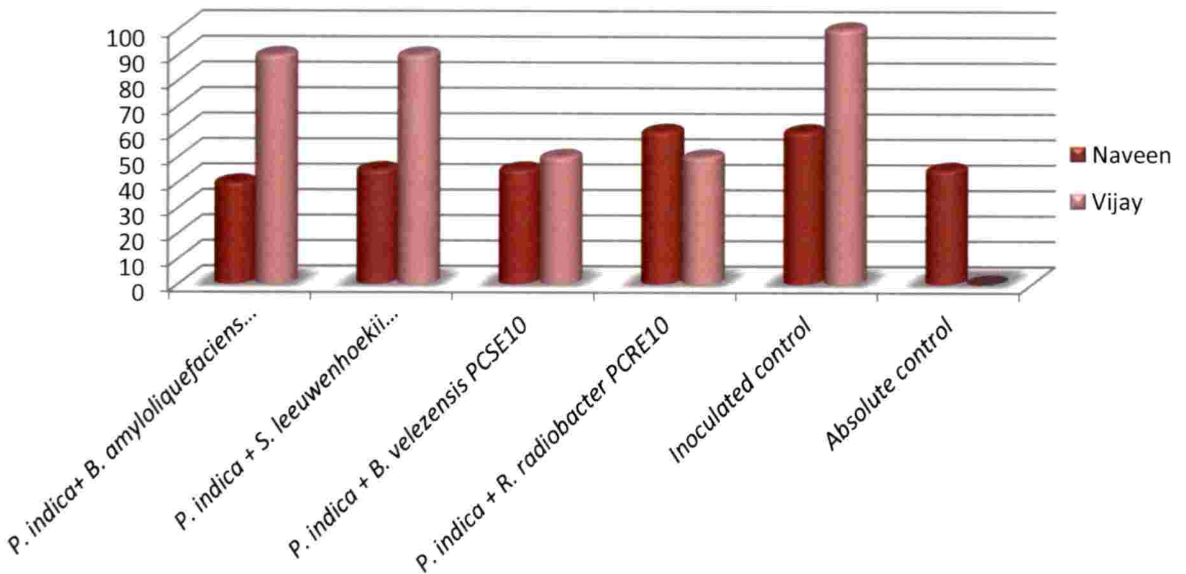


Figure 12. Comparison of disease incidence in the tomato varieties Naveen and Vellayani Vijay treated with combination of bioagents after 21st day of challenge inoculation

compatible with *P. indica.* However in co-culture the bacterial endophyte *B. megaterium* NAT001 did not grow well and therefore the strain was not taken for combined inoculation.

R. radiobacter PCRE10 which showed the highest disease suppression (15%) in the case of Naveen when applied individually, when combined with the fungal endophyte and applied to the nursery plants, showed decreased disease suppression (60%) (Fig 10 to 12). *R. radiobacter* has been reported to be a natural endofungal bacterium within *P. indica* in an earlier report (Sharma, *et al.*, 2008). Whenever the fungal culturing was done, they observed that there was presence of the bacterium along with the fungal culture. The exact effect of the endofungal isolate in the events of interaction between the fungal endophyte and host plants has not been worked out yet. The strain *R. radiobacter* PCRE10 may have some detrimental effect on the positive influence of the fungal endophyte on tomato plants, especially in the bacterial wilt susceptible variety Naveen. However in the variety Vellayani Vijay the disease suppression was similar in individual as well as combined treatments. Varietal difference may have caused this difference.

It was surprising to note that all the combined inoculation resulted in reduced suppression of bacterial wilt incidence in both the varieties except in a few cases (Fig 10 to 12 and Fig 13 to 14). When *B. amyloliquefaciens* VLY24 was combined with *P. indica* in the case of the variety Naveen there was increased disease suppression than the individual treatments (from 55 % to 40 %). The case was same with *S. leeuwenhoekii* KBT004.

When the root colonization pattern of *P. indica* was analyzed it was observed that the maximum root colonization was noticed in the individual application of the fungal bio agent (46.4 %). In all the other cases that involved combination with bacterial bioagents, reduced colonization was observed. This is not surprising as the bacterial bioagents may have some adverse influence on the fungal root colonization,

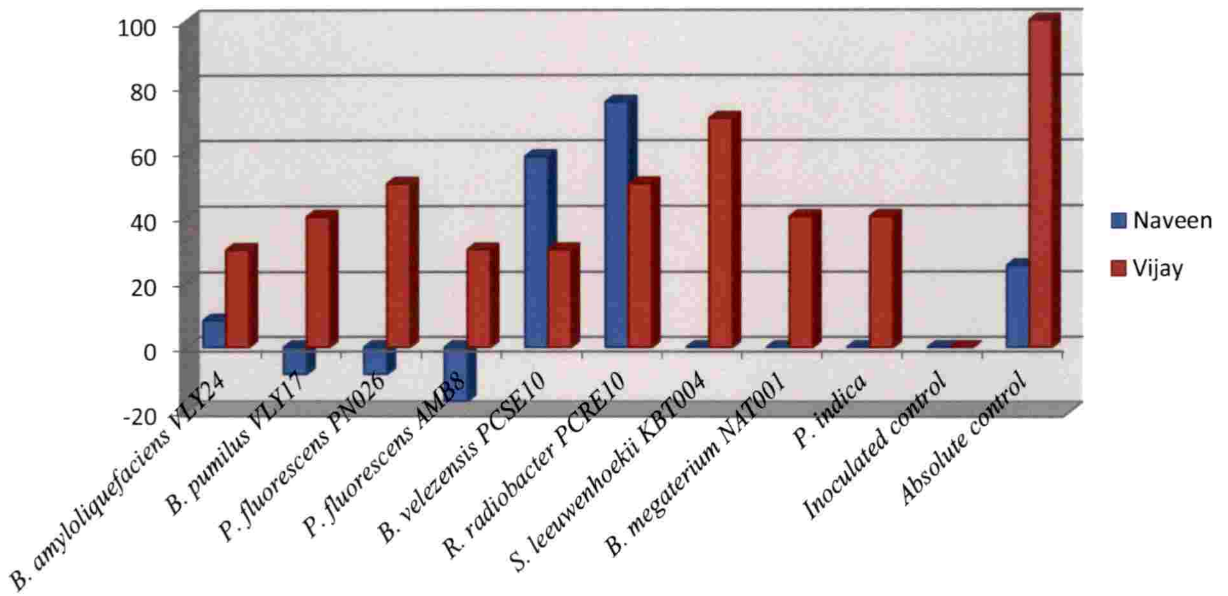


Figure 13. Percentage disease suppression over pathogen inoculated control in the tomato varieties treated with individual bioagents on 21st day after challenge inoculation

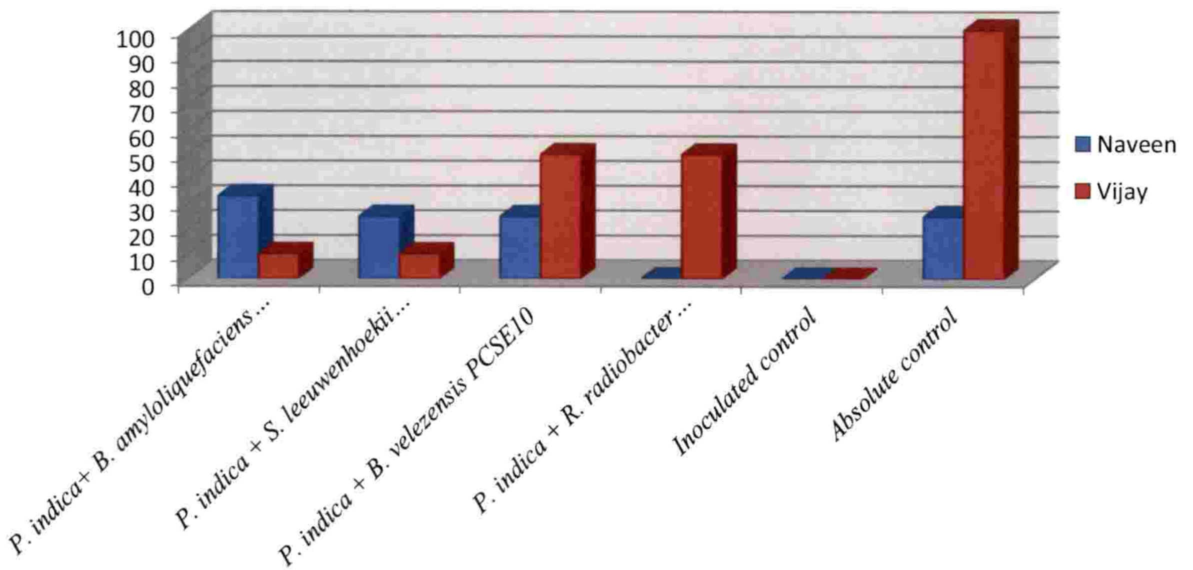


Figure 14. Percentage disease suppression over pathogen inoculated control in the tomato varieties treated with combination of bioagents on 21st day after challenge inoculation

though the same has not been completely prevented. Reduction in the amount of root colonization by *P. indica* in tomato plants when combined inoculation was done along with *B. pumilus* VLY 17 and *P. fluorescens* AMB 8 has already been reported (Varkey, 2018).

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 tomatoes.

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). The survival and root colonization pattern of several rhizobacteria in soil-less transplant medium for tomato has been studied in detail by Yan *et al.*, (2003). In the present study, plant growth promotion by rhizobacteria, bacterial bioagent and fungal endophyte was evaluated in the variety Vellayani Vijay in the nursery stage using individual as well as combinations of bioagents. All the bioagents except the two fluorescent Pseudomonad species were effective in plant growth promotion in the nursery stage (Fig 15-20).

Analysis of biometric observations revealed that plant height, shoot dry weight and root dry weight was the maximum for plants treated with *B. velenzensis* PCSE 10. The same bacterial strain showed an increment in the plant growth when applied in pepper cuttings (Kollakkodan, 2017). Shoot fresh weight and root fresh weight of

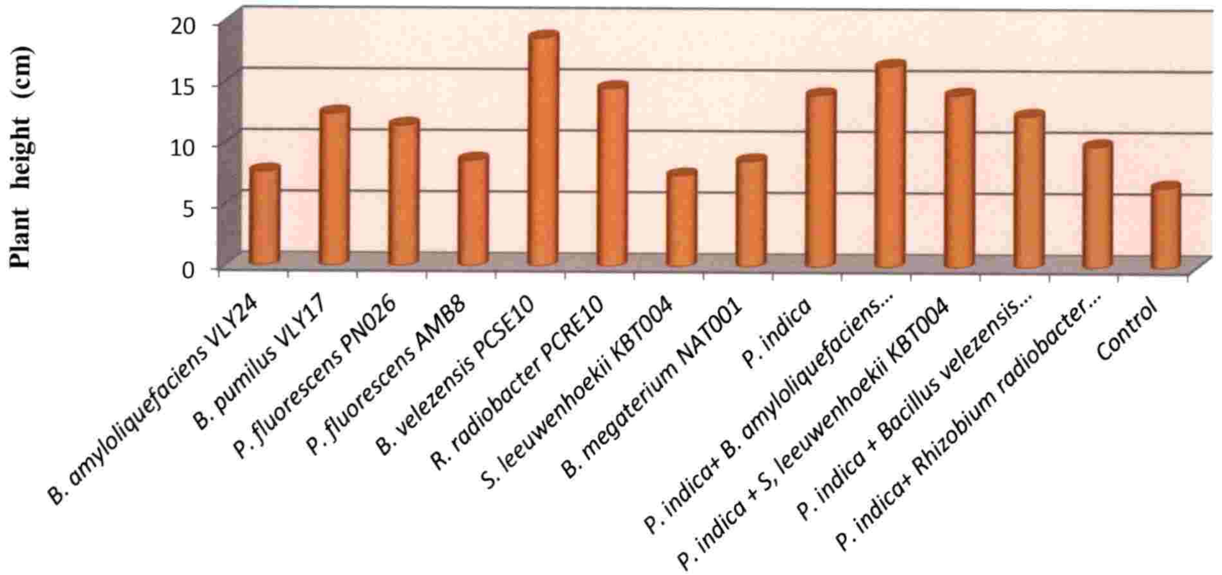


Figure 15. Plant height of the tomato variety Vellayani Vijay on inoculation with the bioagents

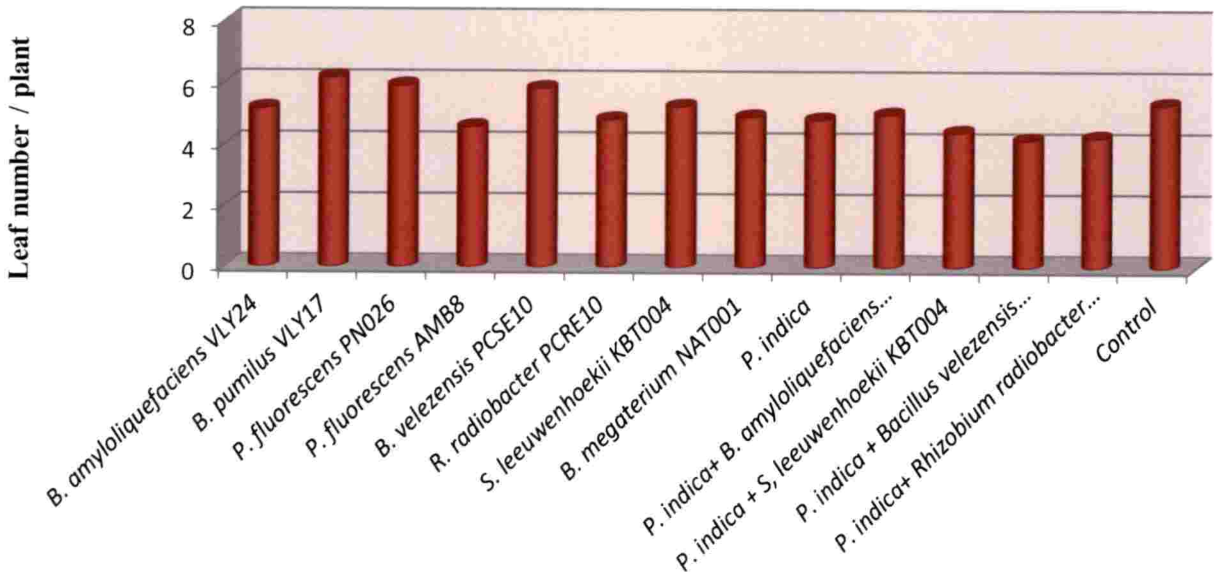


Figure 16. Leaf number of the tomato variety Vellayani Vijay on inoculation with the bioagents

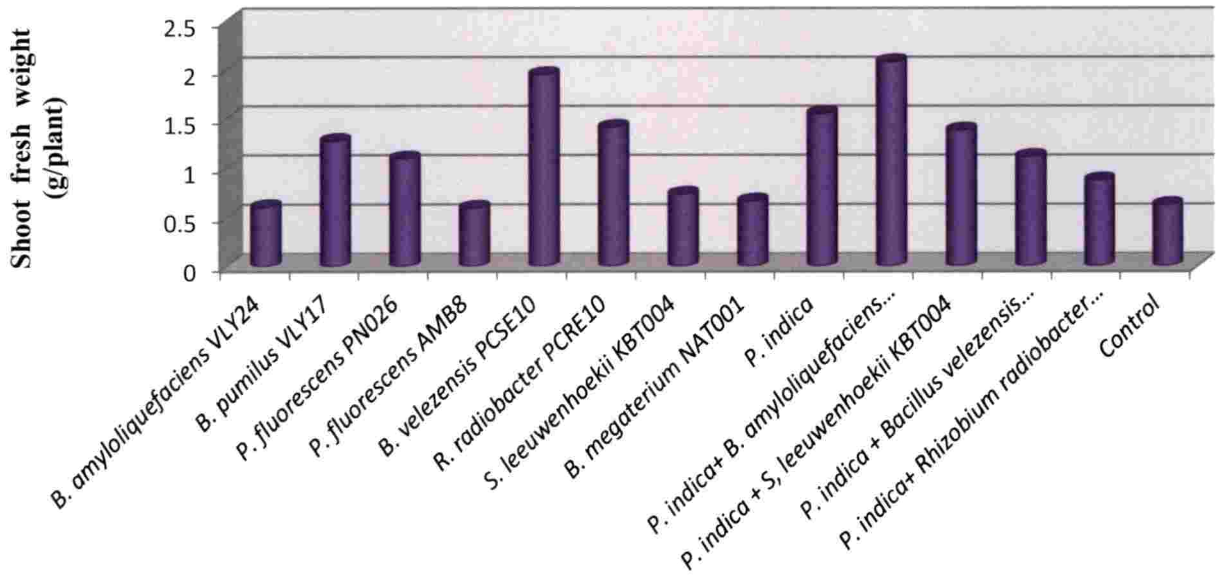


Figure 17. Shoot fresh weight of the tomato variety Vellayani Vijay on inoculation with the bioagents

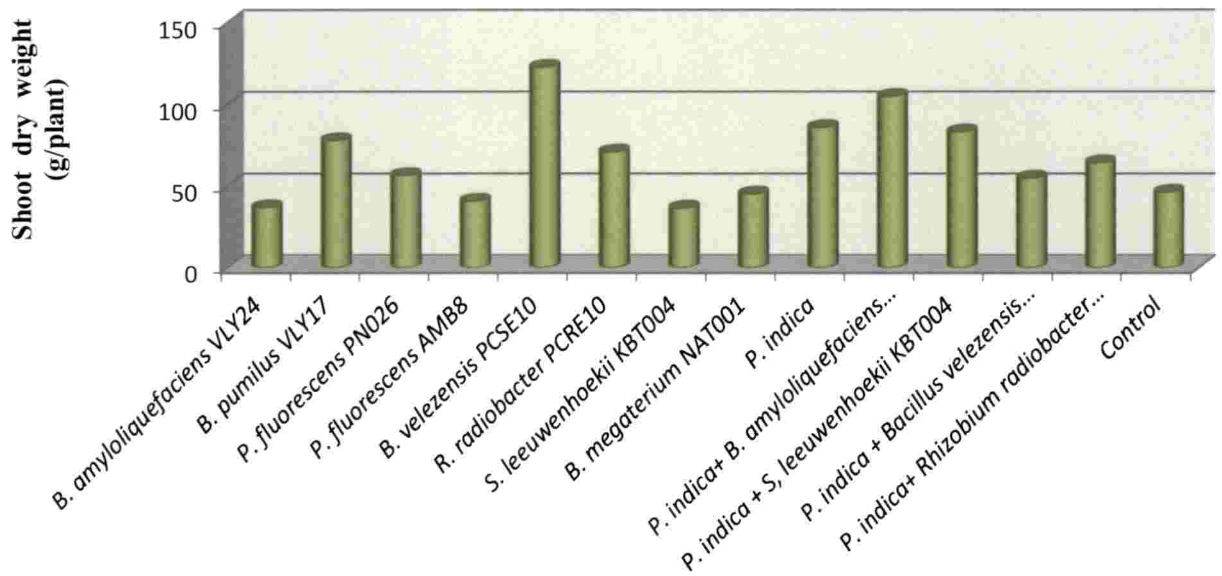


Figure 18. Shoot dry weight of the tomato variety Vellayani Vijay on inoculation with the bioagents

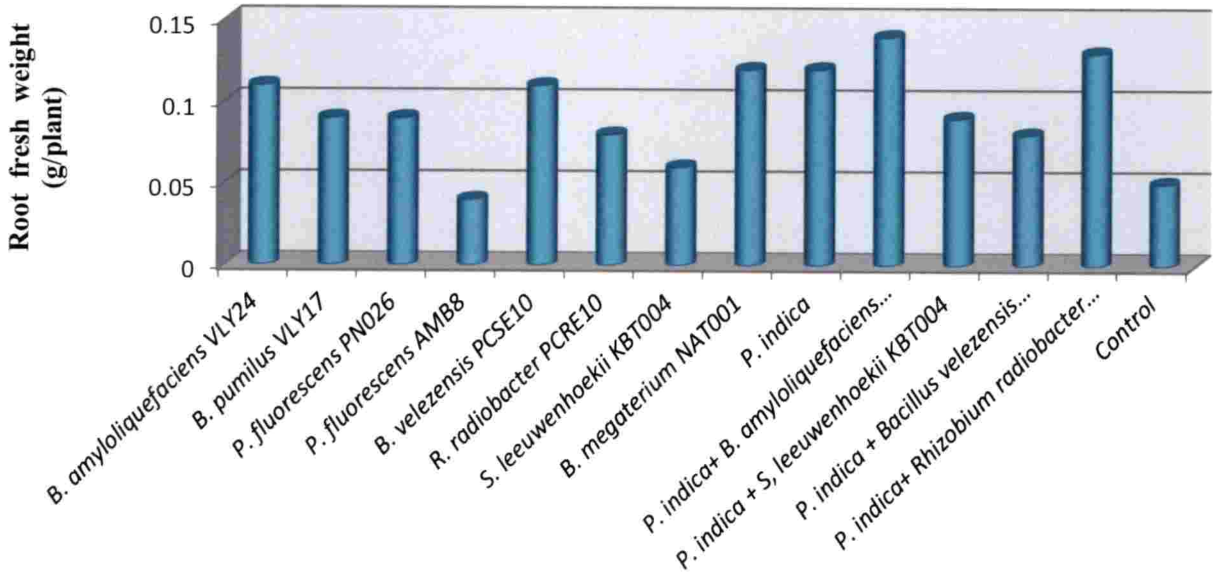


Figure 19. Root fresh weight of the tomato variety Vellayani Vijay on inoculation with the bioagents

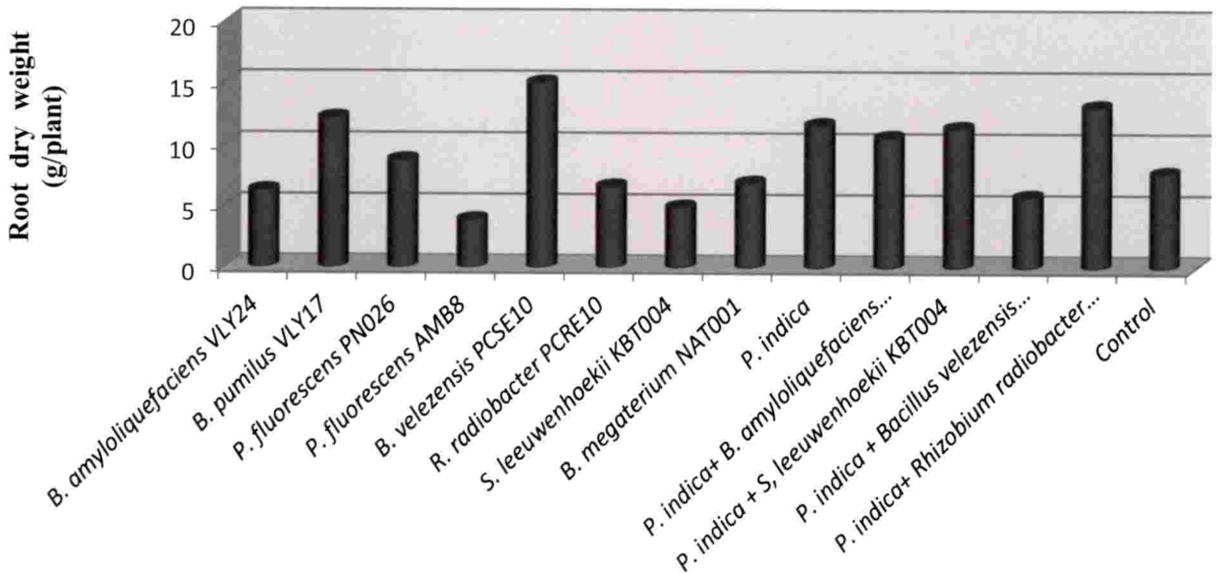


Figure 20. Root dry weight of the tomato variety Vellayani Vijay on inoculation with the bioagents

the plants were enhanced by the combined application of *P. indica* and *B. amyloliquefaciens* VLY 24, whereas leaf number was the maximum for those treated with *B. pumulis* VLY 17. Several of the *Bacillus* spp. would act as biological control agents as well as plant growth promoters. *Bacillus* is an ideal candidate for plant growth promotion and biocontrol of plant pathogens because of its ability to form endospores which are resting structures capable of surviving desiccation, heat, oxidising agents, UV and gamma radiations (Turner and Backman, 1991; Shafi *et al.*, 2017). These attributes endow *Bacillus* an ecological advantage over several other biocontrol bacteria and render it amenable to commercialisation due to the extended shelf life. In addition, it also produces highly stable antibiotics that are detrimental to several pathogens (Baker and Cook, 1974; Handelsman *et al.*, 1990; Osburn *et al.*, 1995; Weller, 1988; Shafi *et al.*, 2017).

Plants inoculated with *P. indica* in combination with *Rhizobium radiobacter* PCRE 10 exhibited significant improvement in major growth parameters. *P. indica* has been reported to have some close association with other bacterial flora. These intimate bacterial species that are present within the hyphae of the fungus included a *Rhizobium radiobacter* strain as well (Sharma *et al.*, 2008). The isolated bacteria from the association also had growth promotional and biological control effect (Glaeser *et al.*, 2016). *Rhizobium radiobacter* PCRE 10 has been reported to be an efficient plant growth promoter and biocontrol bacterium in black pepper also (Kollakkodan, 2017).

Rhizobacteria, endophytic bacteria and the fungal endophyte used in the present study were found to have biocontrol properties against the bacterial wilt pathogen *R. solanacearum.* Besides the biocontrol properties, many of the bioagents had plant growth promotional abilities in the case of tomato in the nursery. Biorational approaches involving rhizobacteria, bacterial endophytes, root endophytic fungus *P. indica* and their combinations may have positive influence on the reduction of incidence of bacterial wilt disease in controlled experimental conditions.

Summary

6. SUMMARY

The investigation entitled "Management of bacterial wilt disease of tomato by the root endophytic fungus *Piriformospora indica*, rhizobacteria and bacterial endophytes" was conducted during 2016-2018 at Department of Agricultural Microbiology, College of Agriculture, Vellayani. Major objective of the study was to assess the potential of root endophytic fungus *Piriformospora indica,* plant growth-promoting rhizobacteria and bacterial endophytes to suppress bacterial wilt incidence in tomato.

Biological control is an economic and environment friendly approach for mitigating plant diseases which mainly involves the use of microbial antagonists to suppress pathogens. Use of bioagents improves the tolerance of crops against biotic and abiotic stresses by triggering its defence capabilities. *R. solanacearum*, the causal organism of bacterial wilt is one among the quarantine pathogens which can survive in soil for a long period thereby detrimentally affecting the tomato production extensively. Conventional methods like physical, chemical and cultural methods were found to be less economic and environmentally unsound compared to biological control, which is emerging as an efficient method for plant disease suppression. The microorganisms residing in the soil as well as in the root zone have been found to be effective in controlling the bacterial wilt pathogen.

The present study evaluated the capacity of rhizobacteria, bacterial endophytes, the root endophytic fungus *P. indica* and their consortium in providing defence against the bacterial wilt pathogen, *R. solanacearum*. The potential of all the bioagents to be used as biological control against *R. solanacearum* was evaluated both under *in vitro* and *in vivo* conditions.

Virulent isolates of bacterial wilt pathogen *R. solanacearum* were isolated from wilt affected tomato plants from the fields of College of Agriculture, Vellayani on selective medium SMSA. The pathogenicity of the strain was proved by artificial inoculation on tomato plants. Population dynamics of the pathogen

was worked out and relationship between optical density of broth culture and population was derived for determining the inoculum density required for carrying out further *in vivo* experiments.

In vitro antagonism shown by the bacterial bioagents against the pathogen was evaluated using both direct and indirect methods. Direct methods employed were cross streak plate assay, agar plug diffusion technique, disc diffusion method and spot on lawn method. *B. amyloliquefaciens* VLY24, *B. velezensis* PCSE10 and *S. leeuwenhoekii* KBT004 were found to have *in vitro* antagonism against *R. solanacearum*. In the cross streak plate assay, largest zone of inhibition was produced by *B. amyloliquefaciens* VLY24 (20 mm) followed by *B. velezensis* PCSE10 (14 mm) and *S. leeuwenhoekii* KBT004 (4 mm). In the other three methods, only the presence or absence of inhibition was analyzed.

Indirect antagonism was checked by agar well diffusion and disc diffusion methods using culture filtrate. Culture filtrate of *B. amyloliquefaciens* VLY24, *B. velezensis* PCSE10 and *P. indica* had antagonistic action against the pathogen. The zone of inhibition was the maximum for *B. velezensis* PCSE10 (7 mm). Cultural filtrate of the endophytic fungus also produced inhibitory effects to a lesser extent with a zone of inhibition of 1 mm.

The bacterial bioagents *R. radiobacter* PCRE10, *B. megaterium* NAT001 and *S. leeuwenhoekii* KBT004 were found to be compatible with the fungal endophyte *P. indica* in the dual culture studies. However, when the compatible bacteria where co-cultured in a single fermentor system along with *P. indica*, it was observed that *B. megaterium* NAT001 failed to grow along with the fungal endophyte. In all other cases, there was increase in population when tested after 24 h of inoculation. Thus, co-culturing of *R. radiobacter* PCRE10 and *S. leeuwenhoekii* KBT004 along with *P. indica* was found to be feasible.

In vivo pot culture experiments were conducted using individual as well as combined application of the bioagents in unsterile soil in field condition. Tomato seedlings were initially raised in pro-trays filled with sterile vermiculite and

perlite (2:1 ratio) as planting medium. Bacterization of the seeds was done with 48 h old culture of bacterial bioagent, 20 minutes prior to seeding. Mycelium of *P. indica* was incorporated into the planting medium at the rate of one per cent (w/v) and filled in the pro-trays. After imposing the treatments, tomato seeds were dibbled into the planting medium. After 21 days, seedlings were transplanted to pots filled with garden soil. Challenge inoculation of the pathogen was done five days after transplanting and weekly observations were recorded for three weeks.

Suppression of bacterial wilt incidence in the susceptible tomato variety Naveen by bioagents was observed. When treatments were applied individually, occurrence of the disease was the minimum in plants treated with *R. radiobacter* PCRE10 (15 percent) after 21 days. The disease suppressive ability of *B. velezensis* PCSE10 (25 percent) was also significantly superior to all other treatments. The un-inoculated control showed disease incidence which could be due to the presence of wilt pathogen in the soil used for planting.

Bacterial bioagents were selected for developing consortium based on their compatibility with the fungal endophyte and inhibitory action against *R. solanacearum*. In the variety Naveen, combined application of *P. indica* and *B. amyloliquefaciens* VLY24 was found to possess the maximum suppressive potential against bacterial wilt incidence.

P. indica was found to successfully colonize the root system of tomato plants upon inoculation. Root colonization pattern by the endophytic fungus on the surviving plants of the variety Naveen registered the highest root colonization of 46.4 percent in plants treated with single application of *P. indica* followed by a combination of *P. indica* and *B. velezensis* PCSE10 (26.77 percent).

Individual application of the bioagents to the moderately tolerant tomato variety Vellayani Vijay suppressed bacterial wilt. *S. leeuwenhoekii* KBT004 (30 percent) exhibited the maximum bacterial wilt suppression after 21 days of challenge inoculation. Lower disease incidence was observed in the combined

application of *P. indica* with *B. velezensis* PCSE10 (50 percent) and *R. radiobacter* PCRE10 (50 percent).

Plant growth promoting ability of the bioagents was tested with the KAU variety Vellayani Vijay in the nursery. Maximum plant height was observed in seedlings raised after seed treatment with *R. radiobacter* PCRE10 (14.49 cm). Maximum leaf number was observed in tomato seedlings treated with *B. pumilus* VLY17 (4.58). Application of *P. indica* with *B. amyloliquefaciens* VLY24 resulted in improved fresh shoot weight (2.08 g plant^{-1}) which was on a par with the plants treated with *B. velezensis* PCSE10 (1.95 g plant^{-1}). However, dry shoot weight was the maximum for plants treated with *B. velezensis* PCSE10 (122.55 mg plant^{-1}) which was on a par with plants treated with a combination of *P. indica* and *B. amyloliquefaciens* VLY24 (104.78 mg plant^{-1}). All the bioagents except *P. fluorescens* AMB8 resulted in improved root growth when applied singly. The highest fresh root weight was recorded in plants treated with a combination of *P. indica* and *B. amyloliquefaciens* VLY24 (0.14 g plant^{-1}). Among the individual treatments, application of *B. velezensis* PCSE10 registered the maximum dry root weight per plant (15.04 mg plant^{-1}) followed by *B. pumilus* VLY17 (12.18 mg plant^{-1}) and the root endophytic fungus. Considering the wilt suppression and plant growth promotion, *R. radiobacter* PCRE10 could be selected as the best treatment among all the tested treatments.

Rhizobacteria, endophytic bacteria and fungal endophyte used in this study were found to have biocontrol properties against the bacterial wilt pathogen, *R. solanacearum.* Apart from the biocontrol property, many of the bioagents had the potential to promote the growth of tomato plants in the nursery. Biorational approaches involving rhizobacteria, bacterial endophytes, root endophytic fungus *P. indica* and their combinations may have positive influence on the reduction of incidence of bacterial wilt disease in controlled experimental conditions.

174425

References

7. REFERENCES

Abbamondi, G.R., Tommonaro., G. and Weyens, N. 2016. Plant growth-promoting effects of rhizospheric and endophytic bacteria associated with different tomato cultivars and new tomato hybrids. *Chem. Biol. Technol. Agric.* 3:112-134.

Abdallah, D.B., Frikha-Gargouri, O., and Tounsi, S. 2018. Rizhospheric competence, plant growth promotion and biocontrol efficacy of *Bacillus amyloliquefaciens subsp. plantarum* strain 32a. *Biological Control.*

Abo-Elyousr, K.A.M., Ibrahim, Y.E., and Balabel, N.M. 2012. Induction of disease defensive enzymes in response to treatment with acibenzolar-S-methyl (ASM) and *Pseudomonas fluorescens* Pf2 and inoculation with *Ralstonia solanacearum* race 3, biovar 2 (phylotype II). *J. Phytopathol.* 160: 382–392.

Achatz, B., von Ruden, S., Andrade, D., Neumann, E., Pons-Kuhnemann, J., Kogel, K.H., Franken, P., and Waller, F. 2010. Root colonization by *Piriformospora indica* enhances grain yield in barley under diverse nutrient regimes by accelerating plant development. *Plant Soil* 333: 59–70.

Adhikari, T.B. and Basnyat, R.C. 1998. Effect of crop rotation and cultivar resistance on bacterial wilt of tomato in Nepal. *Can. J. Plant Pathol.* 20: 283–287.

Aino, M., Tsuchiya, K., Komoto, Y. and Yoshikura, J. 1993. Colonization of tomato root by *Pseudomonas putida* strain FP-16 for the control of bacterial wilt of tomato. *Soil Microorg.* 41: 25-29.

Akiew, E. and Trevorrow, P.R. 1994. Management of bacterial wilt of tobacco. In. Hayward, A. C. and Hartman, G. L. (ed.). Bacterial Wilt: The Disease and Its Causative Agent, *Pseudomonas solanacearum*. Wallingford, UK. pp 179-198.

Aliye, N., Fininsa, C., and Hiskias, Y. 2008. Evaluation of rhizosphere bacterial antagonists for their potential to bioprotect potato (*Solanum tuberosum*) against bacterial wilt (*Ralstonia solanacearum*). *Biol. Control* 47(3): 282-288.

Almaghrabi, O. A., Massoud, S. I., and Abdelmoneim, T. S., 2013. Influence of inoculation with plant growth promoting rhizobacteria (PGPR) on tomato plant growth and nematode reproduction under greenhouse conditions. *Saudi J. Biol. Sci.* 20: 57–61.

Almoneafy, A. A., Kakar, K. U., Nawaz, Z., Li, B., Chun-lan, Y., and Xie, G. L., 2014. Tomato plant growth promotion and antibacterial related-mechanisms of four rhizobacterial *Bacillus* strains against *Ralstonia solanacearum*. *Symbiosis*. 63(2): 59-70.

Amaresan, N., Jayakumar, V., Kumar, K., and Thajuddin, N. 2012. Endophytic bacteria from tomato and chilli, their diversity and antagonistic potential against *Ralstonia solanacearum*. *Arch. Phytopathology Plant Prot.* 45(3): 344-355.

Anith, K. N. 2009. Mature coconut as a bio-fermentor for multiplication of plant growth promoting rhizobacteria. *Curr. Sci.* 10: 1647-1653.

Anith, K. N., Aswini, S., Varkey, S., Radhakrishnan, N. V., and Nair, D. S. 2018. Root colonization by the endophytic fungus *Piriformospora indica* improves growth, yield and piperine content in black pepper (Piper nigurm L.). *Biocatal. Agric. Biotechnol.* 14: 215-220.

Anith, K. N., Faseela, K. M., Archana, P. A., and Prathapan, K. D. 2011. Compatibility of *Piriformospora indica* and *Trichoderma harzianum* as dual inoculants in black pepper (*Piper nigrum L.*). *Symbiosis* 55(1): 11-17.

Anith, K. N., Manomohandas, T. P., Jayarajan, M., Vasanthakumar, K., and Aipe, K.C. 2000. Integration of soil solarization and biological control with a fluorescent *Pseudomonas* sp. for controlling bacterial wilt *Ralstonia solanacearum* of ginger. *J. Biol. Control* 14(1): 25-29.

Anith, K. N., Momol, M. T., Kloepper, J. W., Marois, J. J., Olson, S. M., and Jones, J. B. 2004. Efficacy of plant growth - promoting rhizobacteria, acibenzolar-*S*-methyl, and soil amendment for integrated management of bacterial wilt on tomato. *Plant Dis*. 88: 669-673.

Anith, K. N., Radhakrishnan, N. V., and Manomohandas, T. P. 2003. Screening of antagonistic bacteria for biological control of nursery wilt of black pepper (*Piper nigrum*). *Microbiol. Res.* 158(2): 91.

Anith, K. N., Sreekumar, A., and Sreekumar, J. 2015. The growth of tomato seedlings inoculated with co-cultivated *Piriformospora indica* and *Bacillus pumilus*. *Symbiosis* 65(1): 9-16.

Ansari, M.W., Gill, S. S., and Tuteja, N. 2014, June. *Piriformospora indica* a powerful tool for crop improvement. In. *Proc. Indian Natl. Sci. Acad.* 80: 317-324.

Arora, M., Saxena, P., Abdin, M. Z., and Varma, A. 2018. Interaction between *Piriformospora indica* and *Azotobacter chroococcum* governs better plant physiological and biochemical parameters in Artemisia annua L. plants grown under in vitro conditions. *Symbiosis* 75(2): 103-112.

Arora, M., Saxena, P., Choudhary, D. K., Abdin, M.Z., and Varma, A. 2016. Dual symbiosis between *Piriformospora indica and Azotobacter chroococcum* enhances the artemisinin content in Artemisia annua L. *World J. Microbiol. Biotechnol.* 32(2): 19.

Babu, A. N., Jogaiah, S., Ito, S. I., Nagaraj, A. K., and Tran, L. S. P. 2015. Improvement of growth, fruit weight and early blight disease protection of tomato plants by rhizosphere bacteria is correlated with their beneficial traits and induced biosynthesis of antioxidant peroxidase and polyphenol oxidase. *Plant Sci.* 231: 62-73.

Bagde, U. S., Prasad, R., and Varma, A. 2013. Impact of culture filtrate of *Piriformospora indica* on biomass and biosynthesis of active ingredient aristolochic acid in Aristolochia elegans *Mart. Int. J. Biol.* 6(1): 29.

Baker, K. F. and Cook, R. J. 1974. *Biological control of plant pathogens*. WH Freeman and Company.

Balouiri, M., Sadiki, M., and Ibnsouda, S. K. 2016. Methods for in vitro evaluating antimicrobial activity: A review. *J. Pharm. Analysis*. 6(2): 71-79.

Baptista, M. J., de Souza, R. B., Pereira, W., Lopes, C. A., and Carrijo, O. A., 2007. Effect of soil solarization and biofumigation on tomato bacterial wilt incidence. *Hortic. Bras*. 24: 161–165.

Baptista, M. J., Lopes, C. A., de Souza, R. B., and Furumoto, O. 2006. Effect of soil solarization and biofumigation during autumn on bacterial wilt incidence and potato yield. *Hortic. Bras.* 24: 99–102.

Barazani, O., Von, Dahl. C. C., and Baldwin, I. T. 2007. *Sebacina vermifera* promotes the growth and fitness of *Nicotiana attenuata* by inhibiting ethylene signaling. Plant Physiol. 144: 1223–1232.

Barretti, P. B., de Souza, R. M., Pozza, E. A., and de Souza, J. T., 2012. Combination of endophytic bacteria and resistant cultivars improves control of *Ralstonia* wilt of tomato. Austras. *Plant Pathol*. 41: 189– 195.

Bashan, Y. and de-Bashan, L. E. 2010. How the plant growth-promoting bacterium Azospirillum promotes plant growth–a critical assessment. *Adv. Agron.* 108: 77–136.

Beattie, G. A. 2006. Plant-associated bacteria: survey, molecular phylogeny, genomics and recent advances. In: Gnanamanickam, S. S. (ed) *Plant-Associated Bacteria*. Springer, Netherlands. pp.1–56.

Bertolla, F., Van Gijsegem, F., Nesme, X., and Simonet, P. 1997. Conditions for natural transformation of *Ralstonia solanacearum*. *Appl. Environ. Microbiol.* 63(12): 4965-4968.

Boshou, L. 2005. A broad review and perspective on breeding for resistance to bacterial wilt. In Allen, C., Prior, P., and Hayward, A. C. (ed.), Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. American Phytopathological Society Press, St. Paul, MN. pp. 225-238.

Boukaew, S., Chuenchit, S., and Petcharat, V. 2011. Evaluation of *Streptomyces* spp. for biological control of *Sclerotium* root and stem rot and *Ralstonia* wilt of chili pepper. *BioControl*, 56(3): 365-374.

Buddenhagen, I. and Kelman, A. 1964. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. *Annu. Rev. Phytopathol.* 2: 203-230.

Cao, Y., Pi, H., Chandrangsu, P., Li, Y., Wang, Y., Zhou, H., Xiong, H., Helmann, J. D., and Cai, Y. 2018. Antagonism of Two Plant-Growth Promoting *Bacillus velezensis* Isolates Against *Ralstonia solanacearum* and *Fusarium oxysporum*. *Sci. Rep.* 8(1): 4360p.

Chakravarty, G. and Kalita, M. C. 2012. Biocontrol potential of Pseudomonas fluorescens against bacterial wilt of Brinjal and its possible plant growth promoting effects. *Ann. Biol. Res.* 3(11): 5083-5094.

Champoiseau, P. G., Jones, J. B., and Allen, C. 2009. *Ralstonia solanacearum race 3 biovar 2* causes tropical losses and temperate anxieties. *Plant Health Progress* 10: 1-10.

Chave, M., Crozilhac, P., Deberdt, P., Plouznikoff, K. and Declerck, S., 2017. Rhizophagus irregularis MUCL 41833 transitorily reduces tomato bacterial wilt incidence caused by Ralstonia solanacearum under in vitro conditions. *Mycorrhiza*. 27(7): 719-723.

Chen, Y., Yan, F., Chai, Y., Liu, H., Kolter, R., Losick, R., and Guo, J. H. 2013. Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends on conserved genes mediating biofilm formation. *Environ. Microbiol.* 15(3): 848-864.

Ciampi-Panno, L., Fernandez, C., Bustamante, P., Andrade, N. and Ojeda, S. 1989. Biological control of bacterial wilt of potatoes caused by *Pseudomonas solanacearum*. *Am. Potato J.* 66: 315- 32.

Cook, R. J. 2002. Advances in plant health management in the twentieth century. *Annu. Rev. Phytopathol.* 38: 95–116.

Cortesero, A. M., Stapel, J. O., and Lewis, W. J. 2000. Understanding and manipulating plant attributes to enhance biological control. *Biol. Control* 17(1): 35-49.

Daneshkhah, R., Cabello, S., Rozanska, E., Sobczak, M., Grundler, F. M.W., Wieczorek, K., and Hofmann, J. 2013. *Piriformospora indica* antagonizes cyst nematode infection and development in Arabidopsis roots. *J. Exp. Bot.* 64(12): 3763-3774.

Das, A., Kamal, S., Najam. S. A., Sherameti, I., Oelmuller, R., Dua, M., Tuteja, N., Atul, J. K., and Varma, A. 2012. The root endophyte fungus Piriformospora indica leads to early flowering, higher biomass and altered secondary metabolites of the medicinal plant, *Coleus forskohlii*. *Plant Sig. Behav.* 7:1–10.

De Brito, A. M., Gagne, S., and Antoun, H. 1995. Effect of compost on rhizosphere microflora of the tomato and on the incidence of plant growth-promoting rhizobacteria. *Appl. Environ. Microbiol.* 61(1): 194-199.

Deshmukh, S.and Kogel, K. H. 2007. *Piriformospora indica* protects barley from root rot disease caused by *Fusarium*. *J. Plant Dis. Prot.* 114: 262–268.

Devi, L. R., Menon, M. R., and Aiyer, R. S. 1981. Survival of *Pseudomonas solanacearum* in soil. *Plant Soil* 62: 169-182.

Dittapongpitch, V. and Surat, S. 2003. Detection of Ralstonia solanacearum in soil and weeds from commercial tomato fields using immunocapture and the polymerase chain reaction. *J. Phytopathol.* 151(4): 239-246.

Domenech, J., Reddy, M. S., Kloepper, J.W., Ramos, B., and Gutierrez-Manero, J. 2006. Combined application of the biological product LS213 with Bacillus, Pseudomonas or Chryseobacterium for growth promotion and biological control of soil-borne diseases in pepper and tomato. *BioControl* 51(2): 245-254.

Druege, U., Baltruschat, H., and Franken, P. 2007. *Piriformospora indica* promotes adventitious root formationin cuttings. *Sci. Hortic.* 112: 422–426.

Dukes, P. D., Jenkins, S. F., Jaworski, C. A., and Morton, D. J. 1965. The identification and persistence of an indigenous race of *Pseudomonas solanacearum* in a soil in Georgia. *Plant Dis. Rep.* 49: 586-590.

Elphinstone, J.G. 2005. The current bacterial wilt situation: a global overview. In Allen, C., Prior, P., and Hayward, A. C. (ed.), Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. American Phytopathological Society Press, St. Paul, MN. pp.9-28

Fakhro, A., Andrade-Linares, D. R., Von Bargen, S., Bandte, M., Büttner, C., Grosch, R., Schwarz, D., and Franken, P. 2010. Impact of *Piriformospora indica* on tomato growth and on interaction with fungal and viral pathogens. *Mycorrhiza.* 20: 191-200.

Fegan, M. and Prior, P. 2005. "How complex is the *Ralstonia solanacearum* species complex," in bacterial wilt disease and the *Ralstonia solanacearum* species complex. St. Paul, MN: APS Press. 462p

Feng, H., Li, Y., and Liu, Q., 2013. Endophytic bacterial communities in tomato plants with differential resistance to *Ralstonia solanacearum*. *Afr. J. Microbiol. Res.* 7(15): 1311-1318.

Fortnum, B.A., and Martin, S. B. 1998. Disease management strategies for control of bacterial wilt of tobacco in the southeastern USA. In Prior, P., Allen, C., and Elphinsone, J. (ed.). Bacterial Wilt Disease: Molecular and Ecological Aspects. Springer. Heidelberg, New York. pp 394-402.

Franken, P. 2012. The plant strengthening root endophyte *Piriformospora indica*: potential application and the biology behind. *Appl. Microbiol. Biotechnol.* 96(6): 1455-1464.

Gagne, S., Dehbi, L., Le Quéré, D., Cayer, F., Morin, J. L., Lemay, R., and Fournier, N. 1993. Increase of greenhouse tomato fruit yields by plant growth-promoting rhizobacteria (PGPR) inoculated into the peat-based growing media. *Soil Biol. Biochem.* 25(2): 269-272.

Glaeser, S. P., Imani, J., Alabid, I., Guo, H., Kumar, N., Kämpfer, P., Hardt, M., Blom, J., Goesmann, A., Rothballer, M., and Hartmann, A. 2016. Non-pathogenic Rhizobium radiobacter F4 deploys plant beneficial activity independent of its host *Piriformospora indica*. *The ISME J.* 10(4): 871.

Glick, B. R. 2003. Phytoremediation: synergistic use of plants and bacteria to clean up the environment. *Biotechnol. Adv.* 21:383–93.

Gota, M., 1992. *Fundamentals Of Bacterial Plant Pathology*. Academic Press, San Diego, California, pp: 282-286.

Granada, G. A. and Sequeira, L. 1983. Survival of *Pseudomonas solanacearum* in soil, rhizosphere, and plant roots. *Can. J. Microbiol.* 29: 433-440.

Guo, J. H., Qi, H.Y., Guo, Y. H., Ge, H. L., Gong, L.Y., Zhang, L. X. and Sun, P. H. 2004. Biocontrol of tomato wilt by plant growth-promoting rhizobacteria. *Biol. Control*. 29(1): 66-72.

Handelsman, J., Raffel, S., Mester, E. H., Wunderlich, L., and Grau, C. R. 1990. Biological control of damping-off of alfalfa seedlings with *Bacillus cereus* UW85. *Appl. Environ. Microbiol.* 56(3): 713-718.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W.F. and Kloepper, J.W. 1997. Bacterial endophytes in agricultural crops. *Can J Microbiol.* 43(10):895-914.

Harman, G. E. 2011. Multifunctional fungal plant symbiont: new tools to enhance plant growth and productivity. *New Phytol.* 189: 647–649.

Hayat, R., Ali, S., and Amara, U. 2010. Soil beneficial bacteria and their role in plant growth promotion: a review. *Ann. Microbiol.* 60: 579–98.

Hayward, **A. C.** 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum. Annu. Rev. Phytopathol.* 29: 65–87.

He, L.Y., Sequiera L., and Kelman A. 1983. Characteristics of strains of Pseudomonas solanacearum from China. *Plant Dis.* 67: 1357–1361.

Hong, J. C., Momol, M. T., Pingsheng, J., Stephen, S. M., Colee, J., and Jones, J. B., 2011. Management of bacterial wilt in tomatoes with thymol and acibenzolar-S-methyl. *Crop Prot.* 30: 1340–1345.

Hong, J. K., Jang, S. J., Lee, Y. H., Jo, Y. S., Yun, J. G., Jo, H., Park, C. J., and Kim, H. J. 2018. Reduced bacterial wilt in tomato plants by bactericidal peroxyacetic acid mixture treatment. *The J. Plant Pathol.* 34(1): 78- 92.

Hu, H. Q., Li, X. S., and He, H. 2010. Characterization of an antimicrobial material from a newly isolated Bacillus amyloliquefaciens from mangrove for biocontrol of Capsicum bacterial wilt. *Biol. Control.* 54(3): 359-365.

Hyakumachi, M., Nishimura, M., Arakawa, T., Asano, S., Yoshida, S., Tsushima, S. and Takahashi, H., 2013. *Bacillus thuringiensis* suppresses bacterial wilt disease caused by *Ralstonia solanacearum* with systemic induction of defense-related gene expression in tomato. *Microbes Environ.* 28: 128-134.

James, D. and Mathew, S. 2015. Antagonistic activity of endophytic microorganisms against bacterial wilt disease of tomato. *Int. J. Curr. Adv. Res.* 4(10): 399-404.

Janisiewicz, W. J. 1988. Biocontrol of post harvest diseases of apples with antagonist mixtures. *Phytopathol.* 78: 194-198.

Janisiewicz, W. J. and Bors, B. 1995. Development of a microbial community of bacterial and yeast antagonists to control wound-invading postharvest pathogens of fruits. *Appl. Environ. Microbiol.* 61(9): 3261-3267.

Jetiyanon, K. and Kloepper, J. W. 2002. Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. *Biol. Control* 24(3): .285-291.

Ji, P., Momol, M. T., Olson, S. M., Pradhanang, P. M. and Jones, J. B. 2005. Evaluation of thymol as biofumigant for control of bacterial wilt of tomato under field conditions. *Plant Dis.* 89(5): 497-500.

Ji, X. L., Lu, G. B., Ciai, Y. P., Zheng, C. C., and Mu, Z. M. 2008. Biological control against bacterial wilt and colonization of mulberry by an endophytic *Bacillus subtilis* strains. *FEMS, Microbiol. Ecol.* 65(3): 565-573.

Johri, B.N. 2006. Endophytes to the rescue of plants!. *Curr. Sci. 90*(10): 1315-1316.

Justice, A. H., Faust, J. E., and Kerrigan, J. L., 2018. Evaluating a Novel Method to Introduce a Mycorrhizal-like Fungus, *Piriformospora indica*, via an Inoculated Rooting Substrate to Improve Adventitious Root Formation. *Hort. Technol.* 28(2): 149-153.

Kelman, A. 1953. The bacterial wilt caused by *Pseudomonas solanacearum. Calif. Agric. Exp. Stn. Bull.* 99:1-194.

Khanum, S. A., Shashikanth, S., Umesha, S., and Kavitha. 2005. Synthesis and antimicrobial study of novel heterocyclic compounds from hydroxybenzophenones. *Eur. J. Med. Chem.* 40: 1156–1162.

Kheirandish, Z. and Harighi, B. 2015. Evaluation of bacterial antagonists of *Ralstonia solanacearum*, causal agent of bacterial wilt of potato. *Biol. Control* 86: 14-19.

Kiirika, L. M., Stahl, F., and Wydra, K. 2013. Phenotypic and molecular characterization of resistance induction by single and combined application of chitosan and silicon in tomato against *Ralstonia solanacearum*. *Physiol. Mol. Plant Pathol.* 81: 1–12.

Kirkegaard, J. A., Wong, P. T. W., and Desmarchelier. J. M. 1996. *In vitro* suppression of fungal root pathogens of cereals by *Brassica* tissues. *Plant Pathol.* 45: 593–603.

Kloepper, J.W., Leong, J., Teintze, M. and Schroth, M.N. 1980. Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. *Nature*. *286*: 5776-885.

Kokalis–Burelle, N., Vavrina, C. S., Rosskopf, E. N., and Shelby, R. A. 2002. Field evaluation of plant growth-promoting rhizobacteria amended transplant mixes and soil solarization for tomato and pepper production in Florida. *Plant Soil* 238(2): 257-266.

Kollakkodan, N. 2017. Biocontrol potential of plant associated bacteria from Piper spp. Against Phytophthora capsici infecting black pepper. M.Sc.(Ag) thesis, Kerala Agricultural University, Thrissur, 131p.

Kollakkodan, N., Anith, K. N. and Radhakrishnan, N. V., 2017. Diversity of endophytic bacteria from Piper spp. with antagonistic property against *Phytophthora capsici* causing foot rot disease in black pepper (*Piper nigrum L.*). *J. Tropic. Agric.* 55(1): 63-70.

Kongkiattikajorn, J. and Thepa, S. 2007. Increased tomato yields by heat treatment for controlling *Ralstonia solanacearum*, in soil. Proc. of the 45th Kasetsart University Annual Conference, Kasetsart, Kasetsart University, Bangkok. pp. 450–457.

Kumar, M., Yadav, V., Tuteja, N., and Johri, A. K. 2009. Antioxidantenzyme activities in maize plants colonized with *Piriformospora indica*. *Microbiol.* 155: 780–790.

Kumar, P. and Sood, A. K. 2001. Integration of antagonistic rhizobacteria and soil solarization for the management of bacterial wilt of tomato caused by *Ralstonia solanacearum*. *Indian Phytopathol.* 54(1): 12-15.

Kumar, V., Sarma, M. V. R. K., Saharan, K., Srivastava, R., Kumar, L., Sahai, V., Bisaria, V. S., and Sharma, A. K. 2012. Effect of formulated root endophytic fungus *Piriformospora indica* and plant growth promoting rhizobacteria fluorescent pseudomonads R62 and R81 on *Vigna mungo*. *World J Microbiol. Biotechnol.* 28: 595-603.

Kurabachew, H. and Wydra, K., 2013. Characterization of plant growth promoting rhizobacteria and their potential as bioprotectant against tomato bacterial wilt caused by Ralstonia solanacearum. *Biol. Control* 67(1): 75-83.

Kurabachew, H. and Wydra, K., 2014. Induction of systemic resistance and defense-related enzymes after elicitation of resistance by rhizobacteria and silicon application against *Ralstonia solanacearum* in tomato (*Solanum lycopersicum*). *Crop Prot.* 57: 1-7.

Lachisa, L. and Dabassa, A. 2015. Synergetic effect of rhizosphere bacteria isolates and composted manure on *Fusarium* wilt disease of tomato plants. *Res. J. Microbiol.* 11: 20–27.

Lakshmipriya, P. 2016. *Piriformospora indica* mediated response in taro (*Colocasia esculenta (L.) Schott)* with special emphasis to growth and leaf blight incidence., M.Sc.(Ag) thesis, College of Agriculture, Vellayani.

Lakshmipriya, P., Nath, V. S., Veena, S. S., Anith, K. N., Sreekumar, J., and Jeeva, M. L. 2017. *Piriformospora indica*, a Cultivable Endophyte for Growth Promotion and Disease Management in Taro (*Colocasia esculenta (L.*). *J. Root Crops* 42(2): 107-114.

Lee, Y. H., Choi, C. W., Kim, S. H., Yun, J. G., Chang, S.W., Kim, Y. S. and Hong, J. K., 2012. Chemical pesticides and plant essential oils for disease control of tomato bacterial wilt. J. *Plant Pathol.* 28(1): 32-39.

Lemaga, B., Kakuhenzine, R., Kassa, B., Ewell, P. T., and Priou, S. 2005. Integrated control of potato bacterial wilt in eastern Africa: the experience of African highlands initiative. In Allen, C., Prior, P., and Hayward, A. C. (ed.). Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. American Phytopathological Society Press, St. Paul, MN. pp.145–158.

Lemessa, F. and Zeller, W., 2007. Screening rhizobacteria for biological control of *Ralstonia solanacearum* in Ethiopia. *Biol. Control* 42(3): 336-344.

Li, J.G., and Y.-H. Dong. 2013. Effect of a rock dust amendment on disease severity of tomato bacterial wilt. Antonie Leeuwenhoek 103:11–22.

Li, L., Li, L., Wang, X., Zhu, P., Wu, H., and Qi, S. 2017. Plant growth-promoting endophyte *Piriformospora indica* alleviates salinity stress in *Medicago truncatula*. *Plant Physiol. Biochem.* 119: 211-223.

Lin, W. C., Lu, C. F., Wu, J. W., Cheng, M. L., Lin, Y. M., Yang, N. S., Black, L., Green, K. S., Wang, J. F., and Cheng, C. P. 2004. Transgenic tomato plants

expressing the *Arabidopsis* NPR1 gene display enhanced resistance to a spectrum of fungal and bacterial diseases. *Transgenic Res.* 13: 567–581.

Lin, Y., He, Z., Rosskopf, E. N., Conn, K. L., Powell, C. A., and Lazarovits, G. 2010. A nylon membrane bag assay for determination of the effect of chemicals on soilborne plant pathogens in soil. *Plant Dis*. 94: 201–206.

Lou, Y., Guo, Q., Peng, C., Shi, M. D., Li, H.Y., Li, X., Xue, Q. H., and Lai, H. X. 2018. Effects of three Bacillus strains on growth promoting and rhizosphere soil microflora of tomato. *Ying yong sheng tai xue bao. J. Appl. Ecol.* 29(1): 260-268.

Lugtenberg, B. and Kamilova, F. 2009. Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.* 63: 541-556.

Maji, S. and Chakrabartty, P. K. 2014. Biocontrol of bacterial wilt of tomato caused by *Ralstonia solanacearum* by isolates of plant growth promoting rhizobacteria. *Aust. J. Crop Sci. 8*(2): 208- 224.

Maketon, M., Apisitsantikul, J., and Sirizweekul, C. 2008. Antagonism of *Bacillus subtilis* AP-01 and Trichoderma harzianum AP-001 in controlling tobacco diseases. *Braz. J. Microbiol*. 39(2): 96-300.

Mansotra, P., Sharma, P., and Sharma, S. 2015. Bioaugmentation of *Mesorhizobium cicer*, *Pseudomonas spp.* and *Piriformospora indica* for sustainable chickpea production. *Physiol. Mol. Biol. Plants* 21(3): 385-393.

Mao, L., Wang, Q., Yan, D., Ma, T., Liu, P., Shen, J., Li, Y., Ouyang, C., Guo, M., and Cao, A. 2014. Evaluation of chloropicrin as a soil fumigant against *Ralstonia solanacarum* in ginger (*Zingiber officinale Rosc.*) production in China. *Plos One*, 9(3): 78-84..

Marian, M., Nishioka, T., Koyama, H., Suga, H. and Shimizu, M., 2018. Biocontrol potential of *Ralstonia* sp. TCR112 and Mitsuaria sp. TWR114 against tomato bacterial wilt. *Appl. Soil Ecol.* 128: 71-80.

Martinez-Viveros, O., Jorquera, M. A., Crowley, D. E., Gajardo, G., and Mora, M. L. 2010. Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. *J. Soil Sci. Plant Nutr*. 10: 293–319.

Masunaka, A., Nakaho, K., Sakai, M., Takahashi, H., and Takenaka, S. 2009. Visualization of *Ralstonia solanacearum* cells during biocontrol of bacterial wilt disease in tomato with *Pythium oligandrum. J. Plant Pathol.* 75(4): 281-287.

McLaughlin, R. J. and Sequeira, L. 1988. Evaluation of an avirulent strain of *Pseudomonas solallacearum* for biological control of bacterial wilt of potato. *Am. Potato J.* 65:255-268.

Meena, G, Borkar S. G, and Nisha, M. L. 2015. Population dynamics of plant growth promoting microbes on root surface and rhizosphere of tomato crop and their beneficial effect as bioinoculants on tomato and chilli crop. *Int. J. Adv. Res*. 3: 990–996.

Meena, K. K., Mesapogu, S., Kumar, M., Yandigeri, M. S., Singh, S., and Saxena, A. K. 2010. Co-inoculation of the endophytic fungus *Piriformospora indica* with the phosphate-solunilising bacterium *Pseudomonas striata* affects population dynamics and plant growth in chick pea. *Biol. Fert. Soils* 46: 169-174.

Mena-Violante, H. G. and Olalde-Portugal, V. 2007. Alteration of tomato fruit quality by root inoculation with plant growth-promoting rhizobacteria (PGPR): *Bacillus subtilis* BEB-13bs. *Sci. Hort.* 113(1): 103-106.

Messiha, N. A. S., van Diepeningen, A. D., Farag, N. S., Abdallah, S. A., Janse, J. D., and van Bruggen, A. H. C. 2007. *Stenotrophomonas maltophilia*: a new potential biocontrol agent of *Ralstonia solanacearum*, causal agent of potato brown rot. *Eur. J. Plant Pathol.* 118: 211-225.

Michel, V. V. and Mew, T. W. 1998. Effect of a soil amendment on the survival of *Ralstonia solanacearum* in different soils. *Phytopathol.* 88(4): 300-305.

Murphy, J. F., Reddy, M. S., Ryu, C. M., Kloepper, J. W. and Li, R. 2003. Rhizobacteria-mediated growth promotion of tomato leads to protection against Cucumber mosaic virus. *Phytopathol.* 93(10): 1301-1307.

Nair, C. B. and Anith, N. K. 2009. Efficacy of acibenzolar-S-methyl and rhizobacteria for the management of foliar blight disease of amaranth. *J. Tropic. Agric.* 47(1): 43-47.

Nair, C. B., Anith, K.N., and Sreekumar, J. 2007. Mitigation of growth retardation effect of plant defense activator, acibenzolar-S-methyl, in amaranthus plants by plant growth-promoting rhizobacteria. *World J. Microbiol. Biotechnol.* 23(8): 1183-1187.

Nakaho, K., Inoue, H., Takayama, T., and Miyagawa. H. 2004. Distribution and multiplication of *Ralstonia solanacearum* in tomato plants with resistance derived from different origins. *J. Gen. Plant Pathol.* 70:115–119.

Nakaune, M., Tsukazawa, K., Uga, H., Asamizu, E., Imanishi, S., Matsukura, C., and Ezura, H. 2012. Low sodium chloride priming increases seedling vigor and stress tolerance to *Ralstonia solanacearum* in tomato. *Plant Biotechnol.* 29: 9–18.

Nassal, D., Spohn, M., Eltlbany, N., Jacquiod, S., Smalla, K., Marhan, S., and Kandeler, E. 2018. Effects of phosphorus-mobilizing bacteria on tomato growth and soil microbial activity. *Plant Soil*, 427(1-2): 17-37.

Nawangsih, A. A., Damayanti, I., Wiyono, S. and Kartika, J. G., 2011. Selection and characterization of endophytic bacteria as biocontrol agents of tomato bacterial wilt disease. *Hayati J. Biosci.* 18(2): 66-70.

Nemec, S., Datnoff, L. E., and Strandberg, J. 1996. Efficacy of biocontrol agents in planting mixes to colonize plant roots and control root diseases of vegetables and citrus. *Crop Protect.* 15(8): 735-742.

Nguyen, M. T. and Ranamukhaarachchi, S. L. 2010. Soil-borne antagonists for biological control of bacterial wilt disease caused by *Ralstonia solanacearum* in tomato and pepper. *J. Plant Pathol.* 92(2): 395-406.

NHB [National Horticulture Board]. Indian Horticulture Database. National Horticulture Board, Gurgaon, 2016.

NHB [National Horticulture Board]. Indian Horticulture Database. National Horticulture Board, Gurgaon, 2017.

Nion, Y. A. 2008. Approach to the best control of soil-borne diseases by a combination of biocontrol agents and organic matters. Ph.D thesis.Tokyo University of Agriculture and Technology, Japan. 210p.

Nion, Y. A. and Toyota, K. 2008. Suppression of bacterial wilt of tomato by a *Burkholderia nodosa* strain isolated from Kalimantan soils, Indonesia. *Microbes Environ.* 23: 134–141.

Norman, D. J., Chen, J., Yuen, J. M. F., Mangravita-Novo, A., Byrne, D., and Walsh, L. 2006. Control of bacterial wilt of geranium with phosphorous acid. *Plant Dis.* 90: 798–802.

Norman, D. J., Zapata, M., Gabriel, D.W., Duan, Y. P., Yuen, J. M., Mangravita-Novo, A. and Donahoo, R. S., 2009. Genetic diversity and host range variation of *Ralstonia solanacearum* strains entering North America. *Phytopathol.* 99(9): 1070-1077.

Oelmüller, R., Sherameti, I., Tripathi, S., and Varma, A. 2009. *Piriformospora indica*, a cultivable root endophyte with multiple biotechnological applications. *Symbiosis* 49(1): 1-17.

Osburn, R. M., Milner, J. L., Oplinger, E. S., Smith, R. S., and Handelsman, J. 1995. Effect of *Bacillus cereus* UW85 on the yield of soybean at two field sites in Wisconsin. *Plant Dis.* 79(6): 551-556.

Park, K. S., Paul, D., Kim, Y. K., Nam, K. W., Lee, Y. K., Choi, H.W., and Lee, S.Y., 2007. Induced systemic resistance by *Bacillus vallismortis* EXTN-1 suppressed bacterial wilt in tomato caused by *Ralstonia solanacearum*. *J. Plant. Pathol.* 23(1): 22-25.

Pierson, E. A. and Weller, D. M. 1994. Use of mixtures of fluorescent pseudomonads to suppress take-all and improve growth of wheat. *Phytopathol.* 84: 940-947.

Poussier, S, Vandewalle, P, and Luisetti, J. 1999. Genetic Diversity of African and Worldwide strains of *Ralstonia Solanacearum* as determined by Pcr-Restriction Fragment Length Polymorphism analysis of the Hrp gene region. *Appl. Environ. Microbiol.* 65(5): 2184-2194.

Pradhanang, P. M., Elphinstone, J. G., and Fox, R. T. V. 2000. Identification of crop and weed hosts of *Ralstonia solanacearum biovar 2* in the hills of Nepal. *Plant Pathol.* 49(4): 403-413.

Pradhanang, P. M., Momol, M. T., Olson, S. M. and Jones, J.B., 2003. Effects of plant essential oils on Ralstonia solanacearum population density and bacterial wilt incidence in tomato. *Plant Disease*. 87(4): 423-427.

Prasad, R., Kamal, S., Sharma, P. K., Oelmüller, R., and Varma, A. 2013. Root endophyte *Piriformospora indica* DSM 11827 alters plant morphology, enhances biomass and antioxidant activity of medicinal plant *Bacopa monniera*. *J. Basic Microbiol.* 53(12): 1016-1024.

Prior, P., Bart, S., Leclercq, A., Darrasse, S., and Anais, G. 1996. Resistance to bacterial wilt in tomato as discerned by spread of *Pseudomonas* (*Burholderia*) *solanacearum* in the stem tissues. *Plant Pathol*. 45: 720–726.

Prior, P., Allen, C., and J. Elphinstone 1998. Bacterial wilt disease: molecular and ecological aspects. Springer Verlag, Berlin, Germany.

Priou, S., Gutarra, L. and Aley, P. 2006. An improved enrichment broth for the sensitive detection of Ralstonia solanacearum (biovars 1 and 2A) in soil using DAS–ELISA. *Plant Pathol. 55*(1): 36-45.

Qiang, X., Weiss, M., Kogel, K. H., and Schaefer, P. 2011. Piriformospora indica- A mutualstic basidiomycete with an exceptionally large plant host range. *Mol. Plant Pathol.* 10: 1364-1370.

Quimby, F. C., King, L. R. and Grey, W. E. 2002. Biological control as a means of enhancing the sustainability of crop/land management systems. *Agric. Ecosyst. Environ*. 88: 147–152.

Rabiey, M., Ullah, I., and Shaw, M. W. 2015. The endophytic fungus *Piriformospora indica* protects wheat from *Fusarium* crown rot disease in simulated UK autumn conditions. *Plant Pathol.* 64(5): 1029-1040.

Rai, M. K., Shende, S., and Strasser, R. J. 2008. JIP test for fast fluorescence transients as a rapid and sensitive technique in assessing the effectiveness of arbuscular mycorrhizal fungi in *Zea mays*: analysis of chlorophyll a fluorescence. *Plant Biosyst.* 142: 191–198.

Rai, M. K., Varma, A., and Pandey, A. K., 2004. Antifungal potential of *Spilanthes calva* after inoculation *of Piriformospora indica. Mycoses.* 47(11): 479-481.

Ramesh, R. and Phadke, G. S., 2012. Rhizosphere and endophytic bacteria for the suppression of eggplant wilt caused by *Ralstonia solanacearum. Crop Prot.* 37: 35-41.

Ramesh, R., Joshi, A. A., and Ghanekar, M. P. 2009. Pseudomonads: major antagonistic endophytic bacteria to suppress bacterial wilt pathogen, *Ralstonia solanacearum* in the eggplant (*Solanum melongena L.*). *World J. Microbiol. Biotechnol.* 25(1): 47-55.

Raupach, G. S., and Kloepper, J. W., 1998. Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. *Phytopathol.* 88(11): 1158-1164.

Russo, V. M. 2006. Biological amendment, fertilizer rate, and irrigation frequency for organic bell pepper transplant production. *Hort. Sci.* 41(6): 1402-1407.

Russo, V. M. and Perkins-Veazie, P. 2010. Yield and nutrient content of bell pepper pods from plants developed from seedlings inoculated, or not, with microorganisms. *Hort. Sci.* 45(3): 352-358.

Sangrit, B. C., Techawongstain, S., and Thummabenjapone, P. 2011. Screening tomato cultivars for high β carotene and bacterial wilt resistance. Acta. Hortic. 65-69.

Santos, B. M., Gilreath, J. P., Motis, T. N., Noling, J. W., Jones, J. P., and Norton, J. A. 2006. Comparing methyl bromide alternatives for soilborne disease, nematode and weed management in fresh market tomato. *Crop Prot.* 25: 690–695.

Sarma, M. V. R. K., Kumar, V., Saharan, K., Srivastava, R., Sharma, A. K., Prakash, A., Sahai, V., and Bisaria V. S. 2011. Application of inorganic carrier based formulation of fluorescent pseudomonads and *Piriformospora indica* on tomato plants and evaluation of their efficacy. *J. Appl. Microbiol.* 111: 456-466.

Satheesan, J., Narayanan, A. K., and Sakunthala, M. 2012. Induction of root colonization by *Piriformospora indica* leads to enhanced asiaticoside production in *Centella asiatica. Mycorrhiza* 22(3): 195-202.

Saxena, J., Saini, A., Ravi, I., Chandra, S., and Garg, V. 2015. Consortium of phosphate-solubilizing bacteria and fungi for promotion of growth and yield of chickpea (*Cicer arietinum*). *J. Crop Improve.* 29(3): 353-369.

Schaad, N.W., Jones, J. B., and Chun, W. 2001. *Laboratory guide for the identification of plant pathogenic bacteria* (3rd Ed.). American Phytopathological Society (APS Press), 360p.

Scherf, J. M., Milling, A., and Allen, C. 2010. Moderate temperature fluctuations rapidly reduce the viability of *Ralstonia solanacearum* race 3, biovar 2, in infected geranium, tomato, and potato plants. *Appl. Environ. Microbiol.* 76: 7061–7067.

Schisler, D. A., Slininger, P. A., and Bothast, R. J. 1997. Effects of antagonist cell concentration and two-strain mixtures on biological control of *Fusarium* dry rot of potatoes. *Phytopathol.* 87: 177-183.

Seleim, M. A. A., Saead, F. A., Abd-El-Moneem, K. M. H. and Abo-ELyousr, K. A. M., 2011. Biological control of bacterial wilt of tomato by plant growth promoting rhizobacteria. *Plant Pathol. J.* 10(4): 146-153.

Serfling, A., Wirsel, S. G. R., Lind, V., and Deising, H. B. 2007. Performance of the biocontrol fungus *Piriformospora indica* on wheat under greenhouse and field conditions. *Phytopathol.* 97: 523–531.

Shafi, J., Tian, H., and Ji, M. 2017. *Bacillus species* as versatile weapons for plant pathogens: a review. *Biotechnol. Biotechnol. Equip.* 31(3): 446-459.

Sharafzadeh, S. 2012. Effects of PGPR on growth and nutrients uptake of tomato. *Int. J. Adv. Eng. Technol.* 2(1): 27- 32.

Sharma, J. P. and S. Kumar. 2000. Management of Ralstonia wilt through soil disinfectant, mulch, lime and cakes in tomato (*Lycopersicon esculentum*). *Indian J. Agr. Sci.* 70: 17–19.

Sharma, M., Schmid, M., Rothballer, M., Hause, G., Zuccaro, A., Imani, J., Kämpfer, P., Domann, E., Schäfer, P., Hartmann, A., and Kogel, K.H. 2008. Detection and identification of bacteria intimately associated with fungi of the order Sebacinales. *Cell. Microbiol.* 10(11): 2235-2246.

Shen, M., Xia, D., Yin, Z., Zhao, Q., and Kang, Y., 2018. Bacillus pumilus WP8 exhibits biocontrol efficacy against tomato bacterial wilt via attenuation of the virulence of the pathogenic bacterium. *Soil Plant Sci.* 68(5): 379-387.

Sherameti, I., Shahollari, B., Venus, Y., Altschmied, L., Varma, A., and Oelmuller, R. 2005. The endophytic fungus *Piriformospora indica* stimulates the expression of nitrate reductase and the starch-degrading enzyme glucan-water dikinase in tobacco and *Arabidopsis* roots through a homeodomain

transcription factor that binds to a conserved motif in their promoters. *J. Biol. Chem.* 280: 26241–26247.

Sherameti, I., Tripathi, S., Varma, A., and Oelmüller, R. 2008. The root-colonizing endophyte *Pirifomospora indica* confers drought tolerance in Arabidopsis by stimulating the expression of drought stress–related genes in leaves. *Mol. Plant-Microbe Interact.* 21(6): 799-807.

Singh, A., Singh, A., Kumari, M., Rai, M. K., and Varma, A. 2003. Biotechnological Importance of *Piriformospora indica* Verma et al-A Novel Symbiotic Mycorrhiza-like Fungus: An Overview. *Mol. Plant-Microbe Interact.* 10(4): 699-707.

Slininger, P. J., Schisler, D. A., Shea-Andersh, M. A., Sloan, J. M., Woodell, L. K., Frazier, M. J. and Olsen, N. L. 2010. Multi-strain co-cultures surpass blends for broad spectrum biological control of maladies of potato in storage. *Biocontrol Sci. Technol.* 20: 763-786.

Staley, T. E. and Drahos, D. J. 1994. Marking soil bacteria with *lacZY*. In: Weaver, R. W., Angel, J. S., and Bottomley, P. J. (eds). *Methods of Soil Analysis. Part 2. Microbiological and Biochemical Properties.* Soil Science Society of America, Madison, WI, pp 689–706.

Tan, H., Zhou, S., Deng, Z., He, M. and Cao, L., 2011. Ribosomal-sequence-directed selection for endophytic streptomycete strains antagonistic to *Ralstonia solanacearum* to control tomato bacterial wilt. *Biol. Control*, 59(2): 245-254.

Tan, S., Jiang, Y., Song, S., Huang, J., Ling, N., Xu, Y., and Shen, Q. 2013. Two *Bacillus amyloliquefaciens* strains isolated using the competitive tomato root enrichment method and their effects on suppressing *Ralstonia solanacearum* and promoting tomato plant growth. *Crop Prot.* 43: 134-140.

Trigalet, A. and Trigalet-Demery, D. 1990. Use of avirulent mutants of *Pseudomonas solanacearum* for the biological control of bacterial wilt of tomato plants. *Physiol. Mol. Plant Pathol.* 36: 27- 38.

Turner, J. T. and Backman, P. A. 1991. Factors relating to peanut yield increases after seed treatment with *Bacillus subtilis*. *Plant disease* 75(4): 347-353.

Tusiime, G., Adipala, E., Opio, F., and Bhagsari, A. S. 1998. Weeds as latent hosts of *Ralstonia solanacearum* in highland Uganda: implications to development of an integrated control package for bacterial wilt. Springer, Berlin, Heidelberg, 419p.

Upreti, R. and Thomas, P., 2015. Root-associated bacterial endophytes from *Ralstonia solanacearum* resistant and susceptible tomato cultivars and their pathogen antagonistic effects. *Front. Microbiol.* 6: 255- 272.

van Elsas, J. D., Kastelein, P., van Bekkum, P., van der Wolf, J. M., de Vries, P. M., and van Overbeek, L.S. 2000. Survival of *Ralstonia solanacearum biovar 2,* the causative agent of potato brown rot, in field and microcosm soils in temperate climates. *Phytopathol.* 90(12): 1358-1366.

Vanitha, S. C., Niranjana, S. R., Mortensen, C. N., and Umesha, S. 2009. Bacterial wilt of tomato in Karnataka and its management by *Pseudomonas fluorescens*. *Biocontrol*. 54(5): 685-695.

Varkey, S. 2016. Plant growth-promotion and root knot nematode management in tomato by piriformospora indica and rhizobacteria. M.Sc.(Ag) thesis, Kerala Agricultural University, Thrissur, 149p.

Varkey, S., Anith, K. N., Narayana, R., and Aswini, S. 2018. A consortium of rhizobacteria and fungal endophyte suppress the root-knot nematode parasite in tomato. *Rhizosphere*. 5: 38-42.

Varma, A., Bakshi, M., Lou, B., Hartmann, A., and Oelmueller, R. 2012. Piriformospora indica: a novel plant growth-promoting mycorrhizal fungus. *Agric. Res.* 1(2): 117-131.

Varma, A., Singh, A., Sudha, S. N., Sharma, J., Roy, A., Kumari, M., Rana, D., Thakran, S., Deka, D., Bharti, K., Franken, P., Hurek, T., Blechert, O., Rexer, K. H., Kost, G., Hahn, A., Hock, B., Maier, W., Walter, M., Strack, D., and Kranner, I. 2001. *Piriformospora indica: a cultivable mycorrhiza-like endosymbiotic fungus.* In: Hock B (ed). *Mycota IX.* Springer, Berlin Heidelberg New York. pp125–150.

Varma, A., Verma, S., Sudah, S. N., and Franken, P. 1999. *Piriformospora indica*, a cultivable plant growth-promoting root endophyte. *Appl. Environ. Microbiol.* 65: 2741–2744.

Varma, A., Verma, S., Sahay, N. S., Bu¨tehorn, B., and Franken, P. 1999. *Piriformospora indica,* a cultivable plant-growth-promoting root endophyte. *Appl. Environ. Microbiol.* 65: 2741–2744.

Vasse, J., Frey, P., and Trigalet, A. 1995. Microscopic studies of intercellularinfection and protoxylem invasion of tomato roots by *Pseudomonas solanacearum. Mol. Plant Microbe Interact.* 8: 241–251.

Vavrina, C. S. 1998. Transplant age in vegetable crops. *Hort. Technol.* 8(4): 550-555.

Verma, S., Varma, A., Rexer, K. H., Hassel, A., Kost, G., Sarbhoy, A., Bisen, P., Butehorn, B., and Franken, P. 1998. *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. *Mycologia.* 90: 896–903.

Vidhyasekarn, P. and Muthamilan, M. 1995. Development of formulations of *Pseudomonas fluorescens* for control of chick pea wilt. *Plant Dis.* 79: 782-786.

Vinh, M. T., Tung, T. T., and Quang. H. X. 2005. Primary bacterial wilt study on tomato in vegetable areas of Ho Chi Minch city, Vietnam. In. Allen, C., Prior, P. and Hayward, A. (ed.). Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. American Phytopathological Society Press, St. Paul, MN. pp. 177-184.

Vyshakhi, A. S. 2016. Development of root endophytic plant growth promoters as bio-inoculants for portray seedlings. M.Sc.(Ag) thesis, Kerala Agricultural University, Thrissur, 102p.

Waller, F., Achatz, B., Baltruschat, H., Fodor, J., Becker, K., Fischer, M., Heier, T., Huckelhoven, R., Neumann, C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. *Proc. Natl. Acad. Sci*. USA 102:12286–12291.

Weiss, M., Selosse, M. A., Rexer, K. H., Urban, A., and Oberwinkler, F. 2004. Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. *Mycol. Res.* 108(9): 1003-1010.

Weller, D. M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annu. Rev. Phytopathol*. 26(1): 379-407.

Whipps, J. M. 2001. Microbial interactions and biocontrol in the rhizosphere. *J. Exp. Bot*. 52: 487–511.

Whipps, J. M. and Gerhardson. B. 2007. Biological pesticides for control of seed- and soil-borne plant pathogens. In Van Elsas, J. D., Jansson, J. D., and Trevors, J. T. (ed.).Modern Soil Microbiology, CRC Press, FL. pp.479-501.

Wu, H. X., Wei, A. M., Wang, J., Cao, Y. J., and Xi, G. 2007. Inhibition Effects Of High Voltage Electrostatic Field And Radio Frequency Electromagnetic

Field On The Pathogen Of Bacterial Wilt Of Eucalyptus. J. Nanjing Forestry University, 98p.

Wu, K., Su, L., Fang, Z., Yuan, S., Wang, L., Shen, B., and Shen, Q., 2017. Competitive use of root exudates by *Bacillus amyloliquefaciens* with *Ralstonia solanacearum* decreases the pathogenic population density and effectively controls tomato bacterial wilt. *Sci. Hort.* 218. 132-138.

Yadav, V., Kumar, M., Deep, D. K., Kumar, H., Sharma, R., Tripathi, T., Tuteja, N., Saxena, A. K., and Johri, A. K. 2010. A phosphate transporter from the root endophytic fungus *Piriformospora indica* plays a role in phosphate transport to the host plant. *J. Biol. Chem.* 285:26532–26544

Yamasaki, M., Kusakari, S., Narita, K., Osamura, K., and Nagai, M. 2006. Control of root rot disease of tomatoes by using weak acidic electrolyzed water (WAEW) in the hydroponic culture solution. *J. Antibac. Antifung. Agents* 34: 543–549.

Yamazaki, H. and Hoshina, T. 1995. Calcium nutrition affects resistance of tomato seedlings to bacterial wilt. *Hort. Sci.* 30: 91– 93.

Yamazaki, H., Kikuchi, S., Hoshina, T., and Kimura, T. 2000. Calcium uptake and resistance to bacterial wilt of mutually grafted tomato seedlings. *Soil Sci. Plant Nutr.* 46: 529–534.

Yan, Z., Reddy, M. S., and Kloepper, J. W. 2003. Survival and colonization of rhizobacteria in a tomato transplant system. *Can. J. Microbiol.* 49(6): 383-389.

Yu, J. Q. 1999. Allelopathic suppression of *Pseudomonas solanacearum* infection of tomato (*Lycopersicon esculentum*) in a tomato-chinese chive (*Allium tuberosum*) intercropping system. *J. Chem. Ecol.* 25: 2409–2417.

174425

Yuliar, Y, Nion, Y. A., and Toyota, K. 2015. Recent trends in control methods for bacterial wilt diseases caused by *Ralstonia solanacearum. Microbes. Environ.* 30: 1–11.

Zarea, M. J., Hajinia, S., Karimi, N., Goltapeh, E. M., Rejali, F., and Varma, A. 2012. Effect of *Piriformospora indica* and *Azospirillum* strains from saline or non-saline soil on mitigation of the effects of NaCl. *Soil Biol. Biochem*. 45:139–146.

Zehnder, G.W., Yao, C., Murphy, J. F., Sikora, E. R., Kloepper, J.W., Schuster, D. J., and Polston, J. E. 1999. Microbe-induced resistance against pathogens and herbivores: evidence of effectiveness in agriculture. *Biochem. Ecol. Agric.* 33p.

Zhou, T., Chen, D., Li, C., Sun, Q., Li, L., Liu, F., Shen, Q., and Shen, B. 2012. Isolation and characterization of *Pseudomonas brassicacearum* J12 as an antagonist against *Ralstonia solanacearum* and identification of its antimicrobial components. *Microbiol. Res.* 167(7): 388-394.

Appendices

APPENDIX- I

COMPOSITION OF MEDIA USED

1. Nutrient Agar

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

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

2. King's medium B

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

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

3. **Potato Dextrose Agar**

Peeled and sliced potatoes	- 200g
Dextrose ($C_6H_{12}O_6$)	- 20g
Agar- agar	-20g
Distilled water	- 1000 ml

Potatoes were boiled in 500 ml of distilled water and the extract 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 into the mixture. The volume was made up to 1000 ml with distilled water and medium was sterilized at 15 lbs pressure and 121 °C for 15 min.

4. **Casamino acid-Peptone-Glucose (CPG) medium**

Casamino acid hydrolysate	- 1g
Glucose	- 10g
Peptone	- 10g
Agar- agar	- 20g
Distilled water	-1000 ml

Peptone, Casamino acid hydrolysate and glucose were dissolved in distilled water. Agar- agar was added into this mixture and autoclaved at 15 lbs pressure and 121°C for 15 min.

5. SMSA medium

Manniol	-2.50
L- Glutamic acid	- 1 g
MgSO4.7H2O	- 0.16 g

$MnSO_4 \cdot 7H_2O$	- 0.31 mg
Potassium phosphate monobasic	- 0.27 mg
$ZnSO_4 \cdot 7H_2O$	- 0.55 mg
Ferrous Ammonium Sulphate 6 H_2O	- 0.09 mg
$Cu\ SO_4 \cdot 5H_2O$	- 0.01 mg
Phosphoric acid	- 0.005 mg
KI	- 0.000006 mg
Agar	- 15 g

Supplements:

Tyrothricin	-20 ppm
Cycloheximide	-50 ppm
Bacitracin	-50 ppm
Captan	-10 ppm
Vancomycin	-10 ppm
Chloromycetin	- 0.005 ppm
Penicillin G	- 0.001ppm
TTC solution (1 %)	-10 ml

MANAGEMENT OF BACTERIAL WILT DISEASE OF TOMATO BY THE ROOT ENDOPHYTIC FUNGUS *Piriformospora indica*, RHIZOBACTERIA AND BACTERIAL ENDOPHYTES

by

ATHIRA, S.

(2016-11-128)

ABSTRACT

Submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE IN AGRICULTURE

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF AGRICULTURAL MICROBIOLOGY

COLLEGE OF AGRICULTURE

VELLAYANI, THIRUVANANTHAPURAM-695 522

KERALA, INDIA

2018

ABSTRACT

The study entitled "Management of bacterial wilt disease of tomato by the root endophytic fungus *Piriformospora indica*, rhizobacteria and bacterial endophytes" was conducted during 2016-2018 at Department of Agricultural Microbiology, College of Agriculture, Vellayani with the objective of assessing the potential of root endophytic fungus *Piriformospora indica,* plant growth-promoting rhizobacteria and bacterial endophytes in suppressing bacterial wilt incidence in tomato.

The bacterial wilt pathogen, *Ralstonia solanacearum* was isolated from infected tomato plants on Semi selective medium from South Africa (SMSA). Koch's postulates were proved by artificial inoculation of tomato seedlings. A functional relationship between population and optical density of the broth culture was worked out and was used for determining the inoculum density for challenge inoculation in the biocontrol experiment.

In vitro antagonistic interaction between the bioagents and the pathogen was worked out both by direct and indirect assays. In all direct assays which involved cross streak plating, agar plug diffusion technique, disc diffusion and spot on lawn method, it was found that *Bacillus amyloliquefaciens* VLY24, *Bacillus velezensis* PCSE10 and *Streptomyces leeuwenhoekii* KBT004 exhibited antagonism against *Ralstonia solanacearum*.

Indirect antagonism was checked by agar well diffusion and disc diffusion methods using culture filtrate. Out of the eight bacterial bioagents tested, only two i.e., *Bacillus velezensis* PCSE10 and *Bacillus amyloliquefaciens*VLY24 had inhibitory effect on the pathogen.

Dual culture plate assay on PDA has shown that three bacterial bioagents, *Rhizobium radiobacter* PCRE10, *Bacillus megaterium* NAT001 and *Streptomyces leeuwenhoekii* KBT004 were compatible with *Piriformospora indica*. However, when

the compatible bacteria were co-cultured in a single fermentor system along with *Piriformospora indica* it was observed that *Bacillus megaterium* NAT001 failed to grow along with the fungal endophyte.

Suppression of bacterial wilt incidence by the individual and combined application of bacterial bioagents and fungal endophytes were tested with the wilt susceptible tomato variety Naveen (Indo-American hybrid seeds Pvt. Ltd, Bengaluru) and the moderately tolerant KAU variety Vellayani Vijay. Bioagents were applied during the nursery production of seedlings and the 21 days old seedlings were transplanted to pots filled with unsterile garden soil. Challenge inoculation with the pathogen was done five days after transplanting by drenching the pots with 10 ml each of the bacterial suspension (10^7cfu/ml). The disease incidence was scored at weekly intervals for 21 days.

When the bioagents were tested individually for the suppression of bacterial wilt incidence in the hybrid variety Naveen, maximum disease suppression was observed in plants treated with *Rhizobium radiobacter* PCRE10 (15 percent) after 21 days. The disease suppressive ability of *Bacillus velezensis* PCSE10 (25 percent) was also significantly superior to all other treatments.

Selection of bacterial bioagents for combined application with *Piriformospora indica* was done based on compatibility with the fungal endophyte and inhibitory action against *Ralstonia solanacearum.* Combined application of the fungal endophyte and *Bacillus amyloliquefaciens* VLY24 suppressed the wilt incidence to the highest extent (40 percent).

When bacterial wilt suppression in Vellayani Vijay was tested by the individual application of bacterial bioagents, maximum disease suppression was recorded in plants treated with *Streptomyces leeuwenhoekii* KBT004 (30 percent), when observed 21 days after challenge inoculation. The combination of *Piriformospora indica* with

Rhizobium radiobacter PCRE10 (50 percent) and *Bacillus velezensis* PCSE10 (50 percent) showed significantly lower disease incidence compared to the rest of the treatments.

In the plant growth promotion experiment done with the variety Vellayani Vijay in the nursery stage, maximum plant height was observed in plants treated with *Rhizobium radiobacter* PCRE10 (14.49cm). However, those plants treated with *Bacillus pumilus* VLY17 had the highest number of leaves per plant (4.58). Combined inoculation of *Piriformospora indica* and *Bacillus amyloliquefaciens* VLY24 resulted in improved shoot fresh weight (2.08 g per plant) which was on a par with the plants treated with *Bacillus velezensis* PCSE10 (1.95 g plant^{-1}). However, shoot weight on dry weight basis was the maximum for plants treated with *Bacillus velezensis* PCSE10 (122.55 mg plant^{-1}) which was at par with those treated with combination of *Piriformospora indica* and *Bacillus amyloliquefaciens* VLY24 (104.78 mg plant^{-1}).

The present study revealed that biological management of bacterial wilt in tomato could be a feasible strategy under controlled conditions. The same has to be validated under field conditions before making any recommendations.

174425