ENHANCEMENT OF RESISTANCE TO BACTERIAL WILT IN TOMATO BY ENDOPHYTIC MICROBIAL COMMUNITIES

by

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(2010-21 -109)

THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"ENHANCEMENT OF RESISTANCE TO BACTERIAL WILT IN TOMATO BY ENDOPHYTIC MICROBIAL COMMUNITIES"** is a bonafide record of research work done by me during the course of research and that this thesis has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title of any other University or Society.

Vellanikkara,
Date: 7-2-2015

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CERTIFICATE

Certified that this thesis entitled **"ENHANCEMENT OF RESISTANCE TO BACTERIAL WILT IN TOMATO BY ENDOPHYTIC MICROBIAL COMMUNITIES"** is a record of research work done independently by Mrs. Deepa James 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.

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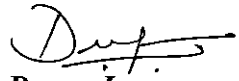

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Introduction

1. INTRODUCTION

Tomato (*Solanum lycopersicum* Mill) is one of the most important vegetable crops grown throughout the world. In fact, it is the fifth important cultivated crop after rice, wheat, maize and potato. The fruits are consumed either fresh or cooked or processed into various products like juice, ketchup, sauce, paste, puree etc. The popularity of tomato is rising among consumers because of its high nutritional value as it contains high levels of vitamins A and C, potassium, phosphorus, magnesium, and calcium. It also contains lycopene and beta-carotene, which are anti-oxidants that promote good health. The high demand for tomato makes it a high value crop that can generate income to farmers. The crop occupies about 7.5 per cent of the total vegetable area in India and its share in the total production is about 8.5 per cent (Indian Horticulture Database, 2011).

Among the various diseases affecting tomato, bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* is the major production limiting factor and it causes extensive losses in Asia and South Pacific regions. The yield loss due to bacterial wilt ranges from 11-93 per cent in India (Kishun, 1987). The warm humid tropical climate and soil conditions prevailing in Kerala are conducive for the occurrence of bacterial wilt and an yield loss up to cent per cent has been reported in susceptible varieties. Bacterial wilt remains a serious and persistent one in Kerala due to variability of the pathogen and the differences in biological and physico-chemical agroecosystem characteristics.

Bacterial wilt is caused by a genetically diverse soil borne pathogen with wide host range and is difficult to manage once it established in the field. Indiscriminate use of chemicals results in environmental pollution, development of resistant strains and detrimental effects on non target organisms including human beings. However, breeding for host resistance has been widely used and has provided some substantial success in Kerala. But it may fluctuate due to the oligogenic nature of the host

resistance, strain variation and aggressiveness of bacterial isolates in different locations. Resistant varieties developed by KAU have recorded a potential yield of 30-33 t/ha only. Moreover, wilt incidence upto 20 per cent has also been observed in some of these varieties. F_1 hybrids with high yield potential of 100 t/ha are available in India, but their cultivation is limited in Kerala, due to their susceptibility to bacterial wilt disease. Hence, it has become necessary to develop a strategy for improving the level of resistance of susceptible/ moderately resistant varieties by inducing systemic resistance.

The focus on the management of plant diseases has been shifted from chemical pesticides to more ecofriendly biopesticides to reduce environmental hazards and minimize the risk of development of pesticide resistant strains of plant pathogens. In recent years, biocontrol has been widely adopted as safe, cost effective and ecofriendly method for the management of diseases. Among the biological management strategies, utilization of antagonistic endophytes is considered as one of the novel approaches for efficient disease management due to their intimate systemic association with the plants. Endophytes are microorganisms that inhabit for at least one period of their lifecycle inside plant tissues without causing any apparent harm to the hosts (Petrini, 1991) and they benefit the host by promoting plant growth and prevent pathogenic organisms from colonization. This novel method of biological control has entered the arena of disease management with attempts to make the plant, defend itself from the pathogens by induced systemic resistance. Therefore, induction of host resistance by endophytic microorganisms has received much attention in recent years as a potential practical method of disease control. The mechanisms involved in the induction of systemic resistance and growth promotion by endophytes have been elucidated in many crops (Benhamou *et al.*, 2000; Rajendran *et al.*, 2006). So it is imperative to explore possibility of identifying effective antagonistic endophytes from tomato for enhancing the defense mechanism against bacterial wilt disease.

Generally, the application of single antagonist leads to inconsistent performance. Recently, a greater thrust is being given for the development of microbial consortium, since it consists of microbes with different biochemical and physiological capabilities, which permit interactions among themselves and will lead to the establishment of a stable and effective microbial community. It will indeed provide better management of diseases by way of synergistic effect and multiple mode of action. Moreover, the consortium approach for disease management has been proved to be successful in certain crops. Recognizing the potentiality of endophytes and the consortial effect of the microorganisms, the present investigation was taken up with the following objectives:

1. Isolation of endophytic microorganisms from tomato plants collected from north, central and south Kerala.
2. *In vitro* and *in planta* screening of endophytes against *R. solanacearum*.
3. Study of mutual compatibility of potential antagonists and development of microbial consortia.
4. Evaluation of microbial consortium in pot culture.
5. Identification of the selected endophytes.
6. Studies on mechanism of antagonism.
7. Effect of secondary metabolites of the selected endophytes on *R. solanacearum.*
8. Studies on induction of systemic resistance in tomato by endophytic microbial consortium.
9. Evaluation of microbial consortium under field condition.

Review of literature

2. REVIEW OF LITERATURE

Tomato is one of the most popular and widely grown vegetables in the world ranking second to potato in many countries. It is rich in pro-vitamin A and vitamin C and contains antioxidant lycopene. The fruits are consumed as fresh or in the processed form, and add variety of colours and flavours to the food. It is a very versatile vegetable for culinary purposes.

2.1. Bacterial wilt disease of tomato

Bacterial wilt disease incited by *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* is one of the most destructive diseases of solanaceous crops in tropics, subtropics, and warm temperate regions of the world. The first report of bacterial wilt of solanaceous crops caused by *Pseudomonas solanacearum* was made by Burril (1890) in connection with an unidentified bacterial disease of potato in United States. Smith (1896) was the first to report bacterial wilt in potato, tomato and brinjal. It is one of the most devastating diseases affecting tomato in the warm humid regions of the world (Buddenhagen and Kelman, 1964).

In India, the occurrence of bacterial wilt of tomato was first reported by Hedayathullah and Saha (1941) from West Bengal. Das and Chathopadhyay (1955) estimated an average reduction in yield of 55-63 per cent due to this disease. Kishun (1987) estimated 10.8 - 92.62 per cent yield loss in tomato due to the disease, in India. In Kerala, the yield loss due to the bacterial wilt incidence ranged from 20 to cent per cent depends upon the varieties (Sadhankumar, 1995). Although it is difficult to estimate total economic losses that cause directly or indirectly by bacterial wilt, it ranks one of the most important plant diseases in the entire world (Gnanamanickam, 2006).

2.2. The pathogen

Pseudomonas solanacearum was identified as the causal agent of bacterial wilt of potato and tomato by Smith (1896). Later Yabuuchi *et al.* (1992) transferred several species of the rRNA homology group II Pseudomonads including *P. solanacearum* to the genus *Burkholderia*. Polyphasic taxonomy and sequencing of 16S rRNA genes revealed dichotomy among the species included in the genus *Burkholderia*. This phylogenetic dichotomy had led to the proposal of new genus *Ralstonia* (Yabuuchi *et al.*, 1995).

The bacterial wilt pathogen, *R. solanacearum* exhibits great degree of both phenotypic and genotypic diversity. Many workers have attempted to group these isolates into biotypes, varieties or races on the basis of difference in physiological characteristics (Kelman, 1954; Buddenhagen and Kelman, 1964; Hayward, 1964). Samaddar *et al.* (1998) identified *R. solanacearum* affecting aubergine, tomato, potato and chilli collected from West Bengal as Race-1. Paul (1998) reported that, among the three *R. solanacearum* isolates obtained from tomato, brinjal and chilli, the isolates of chilli and tomato were identified as Race-1 biovar III and that from brinjal as Race-1 biovar V. Another classification of *R. solanacearum*, based on RFLP and other genetic fingerprinting studies were to Division I (biovars 3,4, and 5 originated from Asia) and Division II (biovars 1, 2A and 2T originated from South America) (Hayward, 2000). Dookun *et al.* (2001) determined the genetic diversity among *R. solanacearum* strains isolated from potato, tomato, beans and anthurium using RFLP techniques. Existence of biovar III, IIIA and V of *R. solanacearum* infecting tomato, brinjal and chilli from different locations of Thrissur and Palakkad districts of Kerala have been reported by Mathew *et al.* (2000) and Mathew (2001). James (2001) grouped the isolates of *R. solanacearum* affecting solanaceous vegetables obtained from Vellanikkara and Kumarakom under Race-1 and those from Ambalavayal under Race-3.

2.3. Management of bacterial wilt disease

2.3.1. Host resistance

The use of resistant variety is a simple, effective and economical means to control soil borne diseases. Breeding for host resistance against *R. solanacearum* has been conducted by a number of workers and numerous reports are available on this field. But it is beyond the scope of present study, to go through the extensive literature on this aspect. This review, is therefore, confined only to the work done at Kerala Agricultural University. KAU reports revealed that, Rajan (1985) introduced the first tomato variety, LE-79 (Sakthi) resistant to bacterial wilt disease. Sadhankumar (1995) evaluated 66 tomato genotypes against bacterial wilt for three seasons and LE-415, LE-79, and LE 79-5 were found to be highly resistant to bacterial wilt.

From the trials conducted at Vellanikkara it was observed that, out of 22 tomato genotypes evaluated, LE-79 (Sakthi), LE 79-5 (Mukthi), LE- 415 (Anagha) and LE-66 (Manulakshmi) were found to be resistant to bacterial wilt (AICVIP, 2002). Another variety, Vellayani Vijay has also been reported to be resistant to this disease (Gopalakrishnan and Devadas, 2014) and these are the five KAU varieties released against bacterial wilt disease.

2.3.2. Chemical control

Attempts have been made by many scientists to study the effect of plant protection chemicals on bacterial wilt pathogen. Campacci *et al.* (1962) reported that, among the various chemicals tested, the bacterium was sensitive to agristrep, streptomycin, penicillin-G-potassic, penicillin procain, dihydrostreptomycin sulphate and erythromycin. The inhibitory effects of streptomycin and streptocycline on *Pseudomonas* have been observed by many workers (Rangarajan and Chakravarthi, 1969; Shivappashetty and Rangaswami, 1971). Jayaprakash (1977) noticed that,

organic amendments combined with antibiotic foliar spray were most effective in controlling bacterial wilt of tomato. Several antibiotics like oxytetracycline, tetracycline, penicillin-G, and streptomycin were found to be inhibitory to the pathogen (Goorani *et al.*, 1978). He *et al.* (1983) opined that, all the strains of *P. solanacearum* from China were susceptible to streptomycin, but resistant to penicillin, viomycin and chloramphenicol. Ishikawa *et al.* (1996) noted that, foliar sprays of validamycin A (250 µg/ml) five days before and two days after inoculation, could reduce the wilt incidence of tomato.

Yamada *et al.* (1997) found that, dazomet combined with soil solarization gave better control of tomato bacterial wilt. Paul (1998) obtained good inhibition and suppression of *R. solanacearum* of tomato with oxytetracycline and streptomycin sulphate. Mazumder (1998) carried out field trials for the control of *R. solanacearum* using Pusa Ruby and observed that, 200 µg/ml of streptomycin treatment was the most effective in controlling disease upto 79 per cent with maximum yields of 274.6 q/ha. He also noticed that, 10 g and 5 g/litre of bleaching powder could also effectively reduce disease incidence and increase the yield.

A perusal of literature revealed that, the reports on fungicidal toxicity on *R. solanacearum* is meagre and scanty. However, attempts have been made to include some of the available literature on the fungicidal action on bacterial wilt pathogen. Severin and Kupferberg (1977) reported that, Bordeaux mixture, copper oxychloride and copper hydroxide were effective in controlling bacterial blight of walnut. Inhibitory action of dithiocarbamate fungicides like nabam, maneb and mancozeb on bacterial wilt pathogen was studied by Goorani *et al.* (1978). Leandro and Zak (1983) noted the inhibitory effect of fungicides including captan, maneb, mancozeb and thiram on *P. solanacearum*. Jyothi (1992) noticed maximum inhibition of *P. solanacearum* with Bordeaux mixture as compared to copper oxychloride and thiride fungicides. The inhibitory effect of copper hydroxide (0.15-0.2%) on bacterial wilt pathogen of tomato was observed by Akbar (2002) and Mathew (2002). The

combination of copper oxychloride (0.2%) + streptocycline (250 ppm), and copper hydroxide (0.2%) were also found very effective in controlling the bacterial wilt disease of solanaceous vegetables in field condition (Mathew, 2004).

2.3.3. Biological control

Biocontrol of plant pathogen is becoming an important component of integrated disease management. In view of the hazardous impact of pesticides and other agrochemicals on the ecosystem, biocontrol of plant diseases as an alternate strategy has received increasing attention in recent years. Therefore, the focus on the management of plant diseases has been shifted from chemical pesticides to more ecofriendly biopesticides to reduce environmental hazards and minimize the risk of development of pesticide resistant strains of plant pathogens. Many bioagents have the potential to reduce crop loss through biocontrol mechanisms.

2.3.3.1. Rhizospheric microorganisms

Effectiveness of biocontrol agents in the management of bacterial wilt disease has been well established. Opina and Valdez (1987) studied the effect of rhizospheric bacteria, *Pseudomonas fluorescens* and *Bacillus polymyxa* against bacterial wilt pathogen of tomato and brinjal and observed significant reduction of the disease with seedling dip method compared to soil drenching.

Anuratha and Gnanamanickam (1990) reported that, the application of *P. fluorescens* could give 95 per cent and 36 per cent protection of tomato plants against bacterial wilt in green house and field condition respectively. In a field trial in Karnataka, seed dressing with bacterinol-100 or *P. fluorescens* was found to be effective for the control of bacterial wilt and increasing the yield (Rao, 1990). Furuya *et al.* (1991) observed the different antibiotic activities of *P. glumae* against *P. solanacearum* depending upon type of media used. All strains of *P. glumae* showed inhibition zones around their colonies on the lawn of *P. solanacearum*. They

also found that, root dipping of tomato seedlings in this bacterial suspension (10^{10} cfu/ml) for 24 h, showed highest disease suppression. Phae *et al.* (1992) noticed suppression of bacterial wilt in heavily infested soil with application of culture suspensions of *Bacillus subtilis* strain NB22.

El-Shanshoury *et al.* (1996) recorded the inhibition of tomato wilt pathogen by two *Streptomyces* spp. under *in vitro* condition. Silveira *et al.* (1996) observed the inhibitory effect of a number of microorganisms including *Streptomyces griseochromogenus*, *S. griseus*, *P. fluorescens*, *Trichoderma pseudokoningii*, *P. aeruginosa*, *B. coagulans*, *B. megaterium* and *B. cereus* against *R. solanacearum* under *in vitro* condition. Das and Bora (2000) reported that, among the different biocontrol agents, *P. fluorescens, B. subtilis, T. harzianum, T. viride, T. koningii, Aspergillus terreus* and *Gliocladium virens,* evaluated under *in vitro* and *in vivo*, the application of *P. fluorescens* showed least wilt incidence in tomato and pre-inoculation of antagonists was highly effective in disease suppression compared to co-inoculation and post- inoculation methods. According to Akbar (2002), seed treatment + soil drenching with *P. aeruginosa* was found effective in reducing the wilt incidence to 11.1 per cent in highly susceptible tomato variety, Pusa Ruby. Manimala (2003) and Mathew (2004) reported the antagonistic effect of *T. viride, T. pseudokoningii, T. harzianum, T. virens, Aspergillus niger, P. aeruginosa, P. fluorescens,* and *B. subtilis* against *R. solanacearum* causing bacterial wilt in solanaceous vegetables.

Seleim *et al.* (2011) tested the efficacy of plant growth promoting rhizobacteria (PGPR) including *P. fluorescens*, *P. putida, B. subtilis* and *Enterobacter aerogenes* against *R. solanacearum* under *in vitro* and *in vivo* condition and observed that, *P. fluorescens* exhibited the lowest wilt incidence in tomato, followed by *P. putida* and *B. subtilis* under greenhouse conditions. Maji and Chakrabartty (2014) reported that, among the PGPR isolated from tomato field, *P. aeruginosa* (T-1), *Pseudomonas* sp. (BH-25), *Pseudomonas* sp. (AM-12),

Pseudomonas sp. (AM-13) and *P. putida* (R-6), the strain *Pseudomonas* sp. (BH-25) was found to be highly effective against *R. solanacearum* and all the strains showed plant growth promotion activity.

2.4. Concept of endophytes

A novel method of biological control using endophytes has entered the arena of disease management with attempts to make the plant, defend itself from the pathogens. The beneficial effects that the endophytes can confer on plants have made their role highly significant in biological control of diseases in various crops (Bargabus *et al.*, 2004; Kloepper *et al.*, 2004).

The term 'endophyte' is derived from two Greek words, 'endon' meaning 'within' and 'phyton' meaning 'plant'. Endophytes have been defined in several ways and the definitions have been modified as the research in this field advanced. The earliest definition was given by De Bary (1866) as the microorganisms that colonize internal plant tissues. Perotti (1926) reported the presence of non pathogenic microorganisms in plant tissues for the first time. Caroll (1986) defined endophytes as asymptomatic microorganisms living inside plant tissues. According to Petrini (1991), endophytes are the microorganisms that inhabit for at least one period of their life cycle inside plant tissues without causing any apparent harm to the hosts. Gordon and Okamoto (1992) observed that, symptomless internal colonization of healthy tissues by microorganisms is a wide spread and well documented phenomenon. They may originate from indigenous species that occur either naturally in soil or may be introduced through various agricultural practices. Endophytes also constitute a valuable source of secondary metabolites for the discovery of new potential therapeutic drugs (Miller, 1995). Secondary metabolites such as alkaloids, terpenoids, sterols and phenolic compounds are the constituents which play a major role in plant defense mechanisms (Nicholson and Hammerschmidt, 1992; Kuc, 1995). Some of the

endophytic bacterial strains have been reported to produce metabolites which can play an important role in ISR against many plant diseases (Mavrodi *et al.*, 2001).

Schulz and Boyle (2005) use the term "endophyte" to describe those bacteria and fungi that can be detected at a particular moment within the tissues of apparently healthy plants. These endophytic bacteria are ubiquitous in most plant species influencing the host fitness by disease suppression, contaminant degradation and plant growth promotion (Kuklinsky-Sobral *et al.*, 2005). According to Azevedo and Araujo (2007), endophytes are microbes that inhabit the interior of plant tissues causing no harm to the host and those do not develop external structures, excluding this way the nodulating bacteria and mycorrhizal fungi. Inside the host, endophytes have greater access to nutrients and a comfortable habitual niche (Ting *et al.*, 2008).

2.5. Isolation of endophytes

The isolation procedure is of key importance for research with endophytes. Different methods have been adopted for isolation of endophytic microorganisms *viz.* homogenization/trituration and vacuum methods. Gardner *et al.* (1982) had observed the better efficiency of homogenization of both root and twig of citrus compared with vacuum methods. In the protocol for isolation of endophytes described by Petrini (1986), the leaf and stem samples were cut into 5-7 mm pieces after surface sterilization, and directly transferred to plates containing PDA.

Bell *et al.* (1995) compared the efficiency of vacuum extraction with trituration method for isolation of endophytes from grape vine and they obtained more number of colonies of endophytic bacteria by homogenization than by vacuum extraction method. McInroy and Kloepper (1995) used trituration technique for the isolation of endophytic bacteria from sweet corn and cotton.

Hallman *et al.* (1997) considered trituration technique to be ideal for the isolation of endophytic bacterial population from vascular tissue. Surface sterilized leaf/stem/root bits were homogenized into a fine paste with suitable sterile buffer in a

sterilized mortar and pestle under aseptic conditions and then serially diluted in the same buffer and plated on appropriate medium. Zinniel *et al.* (2002) adopted similar method for isolating endophytic bacteria from soybean, wheat and prairie plants. However, trituration technique is the most popular method as it yielded maximum endophyte count (Rai *et al.*, 2007).

An attempt was made by Haiyan *et al.* (2005) for the isolation of endophytic fungi from medicinal plants in which the samples were surface sterilized by sequentially dipping in 0.5 per cent sodium hypochlorite (2 min) and 70 per cent ethanol (2 min), rinsed with sterile water, and sterilized samples were placed on PDA medium and incubated at 25°C. Mejia *et al.* (2008) isolated endophytes from cocoa leaves by washing in running tap water followed by surface sterilisation in 0.5 per cent sodium hypochlorite for three minutes and 70 per cent ethanol for two minutes then immersed in sterile water for one minute; and placed on 2 per cent malt extract agar. Shi *et al.* (2009) isolated endophytic microorganisms from sugar beet after surface sterilization with 70 per cent ethanol and 1.05 per cent solution of commercial bleach followed by rinsing in sterile phosphate buffer. Sterility checks were maintained to confirm no chances of surface contaminants.

Trituration technique was adopted for the isolation of endophytes after surface sterilization with disinfestants like sodium hypochlorite (Fisher *et al.*, 1992), ethanol (Dong *et al.*, 1994) mercuric chloride (Sriskandarajah *et al.*, 1993), hydrogen peroxide (McInroy and Kloepper, 1995) or a combination of two or more of these disinfestants (Pleban *et al.*, 1995). The efficacy of surface sterilization was confirmed by maintaining sterility checks and the absence of growth of any microorganism on the medium confirmed the proper surface sterilization procedure (Schulz *et al.*, 1993; Hallman *et al.*, 1997).

The dilution factors used for the isolation of endophytes vary with different plant species. Mathew (2007), Uppala (2007), and Balan (2009) isolated bacterial and

fungal endophytes with 10^{-2} dilution. Nawangsih *et al.* (2011) reported that, dilutions upto 10^{-5} have been used for the isolation of endophytic bacteria from stem. Nandhini *et al.* (2012) used 10^{-10} dilution for the bacterial isolation from stem and root of tomato. Various workers observed 10^{-1} dilution being the ideal for the isolation of endophytic actinomycetes (Cao *et al.*, 2005; Tan *et al.*, 2006; Sreeja, 2011).

2.6. Occurrence and diversity of endophytes

2.6.1. Endophytic bacteria

Perotti (1926) was the first to describe the occurrence of non pathogenic flora in root tissues. The diversity and distribution of endophytic bacteria was first observed by Gardner *et al.* (1982), who identified bacteria present in the xylem fluid from the roots of the rough lemon rootstock and among the 13 genera, the most frequently occurred ones were *Pseudomonas* (40%) and *Enterobacter* (18%), and were regarded as the dominant ones, while the others were classified as rare species.

Jacobs *et al.* (1985) reported seven bacterial genera such as *B. subtilis, Erwinia herbicola, P. aeruginosa, P. fluorescens, Corynebacterium* sp., *Lactobacillus s*p. and *Xanthomonas* sp. from healthy sugar beet root tissue. Fisher *et al.* (1992) isolated both Gram negative and positive bacteria (including *B. subtilis*) from maize. In most of the studies, bacteria have been isolated from the crop plants like sugarcane (Dong *et al.*, 1994), sweet corn (McInroy and Kloepper, 1995), *Zea mays* L and *Zea luxurians* (Palus *et al.*, 1996; Chelius and Triplett, 2000), rice (Barraquio *et al.*, 1997) cotton (Quadt-Hallmann *et al.*, 1997) and potato (Sturz *et al.*, 1998). Ryan *et al.* (2008) also supported the fact that, endophytic bacteria in a single plant host are not restricted to a single species but comprise of several genera and species.

The population density of endophytic bacteria is highly variable, depending on the species, host genotype, the host developmental stage, and inoculum density

(Pillay and Nowak, 1997). In general, endophytic bacteria occur at lower population densities than rhizospheric bacteria and endophytic populations are better protected from biotic and abiotic stresses than rhizospheric bacteria (Hallman *et al.*, 1997). Tan *et al.* (2003) also opined that, the population of endophytes vary among plants. Rosenblueth and Martínez-Romero (2004) found that, endophytes occur in lesser population compared to rhizosphere bacteria.

Yang *et al.* (2011) recorded 72 endophytic bacteria from tomato, of which 45 strains from stems and 27 strains from leaves and found that, the isolation efficiency of bacteria from stems was higher than from leaves. Patel *et al.* (2012) isolated and characterized 18 bacterial endophytes from root and stem of tomato plants collected from different regions of Gujarat.

Recent studies showed significant difference in endophytic colonization and the type of endophytes between root, stem, and leaf tissues (Lodge *et al.*, 1996). Mostly, roots have the high population of endophytes when compared to stem and leaves (Rosenblueth and Martínez-Romero, 2004). Mathew (2006a) isolated *P. fluorescens* and *P. stutzeri* from roots of ginger effective against *R. solanacearum* of ginger and chilli and *Pythium aphanidermatum* of ginger. An antagonistic endophyte *B. megaterium* from black pepper was found to be effective against *Phytophthora capsici* of black pepper and *R. solanacearum* of chilli. (Mathew, 2006b). Endophytic bacteria were isolated from both roots and stems of sugarcane plants by Mendes *et al.* (2007) with a significantly higher density in the roots. Similarly, Uppala (2007) isolated endophytes from roots and stems of amaranth with maximum in roots. According to Shankar-Naik *et al.* (2009), the extent of colonization of dominant endophytes in rice was more in roots (30.23%) than leaves (17.24%). Balan (2009) isolated more number of endophytes from roots than from petiole and leaves of anthurium.

2.6.2. Endophytic fungi

Recent studies have revealed the ubiquity of the endophytic fungi residing within the plants. Rodrigues and Samuels (1990) isolated, eleven species of endophytic fungi from a tropical palm tree growing in the rainforest of Queensland, Australia with Xylariaceous fungi being the predominant ones. Rodrigues (1994) prepared a review on endophytic communities of palm leaves, mainly from *Euterpe oleracea* according to which the most common endophytes were *Aspergillus, Phomopsis, Wardomyces,* and *Penicillium*. Sixteen endophytic fungal taxa were isolated from banana, among which *Xylaria* sp. was the most frequent one followed by *Colletotrichum musae* and *Cordana musae* (Pereira *et al.*, 1999). Studies of endophytic fungi in woody plants showed that, they are highly abundant and diverse, particularly in the tropics (Arnold *et al.*, 2000). Most endophytes isolated to date have been ascomycetes and their anamorphs; however Rungjindamai *et al.* (2008) reported several endophytes as basidiomycetes and the colonization and isolation rates of endophytic fungi from plants varied greatly.

Samuels *et al.* (2000) reported endophytic *T. stromaticum* against cocoa witches' broom pathogen. Gamboa *et al.* (2002) indicated that, the predominant endophytic genera found in all tropical plant species surveyed were *Xylaria*, *Colletotrichum*, and *Phomopsis*. Mathew (2006b) isolated *T. viride* and *T. pseudokoningii* from black pepper against *P. capsici*. Uppala (2007) observed the presence of *Aspergillus* spp, *Penicillium* spp and *T. harzianum* as endophytic fungi in red and green amaranth. Bailey *et al.* (2008) reported that, *Trichoderma* sp. can persist not only in the rhizosphere but also within the tissues forming endophytic associations with cacao plant. Mejia *et al.* (2008) reported endophytic *Trichoderma* spp. as biocontrol agent against *Moniliophthora roreri* in cacao. Endophytic *Trichoderma* isolates obtained from tropical environments were found to delay disease onset and induce resistance against *P. capsici* in hot pepper (Bae *et al.*, 2011).

Kurian (2011) obtained *Penicillium minioluteum* as promising endophytic fungus from cacao against Phytophthora pod rot. Xia *et al.* (2011) reported the distribution of various species of endophytic and epiphytic *Trichoderma* in banana roots with the largest population comprised of *T. asperellum*, *T. virens*, and *Hypocrea lixii*.

2.6.3. Endophytic actinomycetes

Castillo *et al.* (2002) isolated endophytic actinomycetes from stem and root of tomato, banana and wheat. Cao *et al.* (2004) obtained 240 actinomycetes strains from the internal tissues of leaves and roots of healthy and wilted banana plants. Teng *et al.* (2006) isolated endophytic actinomycetes *Chrysobacterium* sp. and two *Streptomyces* sp. from tomato plants. Jiefeng *et al.* (2009) obtained 44 endophytic actinomycetes from tomato, pepper and egg plant.

Nimnoi *et al.* (2010) used PCR denaturing gradient gene electrophoresis to determine diversity and community of endophytic actinomycetes distributed within the roots of eagle wood and confirmed the presence of endophytic actinomycetes of genera *Nocardia, Pseudonocardia, Streptomyces* and *Actinomadura* within the roots. Sreeja (2011) obtained five isolates of endophytic actinomycetes of tomato from Thrissur and Palakkad districts of Kerala, which belonged to genus *Streptomyces*.

2.7. Endophytes in plant protection and plant growth promotion

Endophytic microbes have several attributes which make them attractive as potential biocontrol agents. They colonize and form associations within plant tissues without causing disease, and these are protected from variable environmental conditions and competition for limited nutrients and they make use of plant sap as their medium of multiplication (Azevedo *et al.*, 2000). Many studies have been carried out revealing the effectiveness of endophytes in plant disease management.

Studies have been conducted on the plant growth promoting abilities of various endophytic microorganisms. They promote plant growth through the

improved cycling of nutrients and minerals such as nitrogen, phosphate and other nutrients. These include phosphate solubilisation activity (Verma *et al.*, 2001; Wakelin *et al.*, 2004) and indole acetic acid production (Lee *et al.*, 2004). According to Vijayaraghavan (2007), PGPR isolates including *Bacillus* spp and *Pseudomonas* spp produced indole acetic acid in varying levels.

2.7.1. Endophytic bacteria

The role of endophytic bacteria in plant disease management have been well studied. Chen *et al.* (1995) reported the role of endophytic bacteria *P. putida, P. corrugata* and *Bacillus pumilus* in the control of *R.* wilt of cotton. According to Pleban *et al.* (1995), the endophytic bacteria have brought about significant control of *R. solani* in cotton and *Sclerotium rolfsii* in beans. Similar results have also been reported by Krishnamurthy and Gnanamanickam (1997) in rice. Bacterial endophytes have also been reported to enhance resistance against *Fusarium* wilt in tomato (M'Piga *et al.*, 1997).

The role of endophytic bacteria in reducing or preventing the deleterious effects of phytopathogenic organisms have been reported by many workers. Sturz *et al.* (1999) reported inhibitory effect of endophytic bacteria from potato tubers against *P. infestans*. The inhibitory effect of endophytic bacterial strains 73a and A1a on *Verticillium dahliae* of cotton was noticed by Fu *et al.* (1999. Viswanathan *et al.* (2003) observed *in vitro* antagonism among the endophytic isolates, *P. aeruginosa, P. fluorescens* and *P. putida* on the red rot pathogen of sugarcane, *C. falcatum,* and also noticed reduction in the red rot disease by application of endophytic strain of *P. fluorescens.*

Nejad and Johnson (2000) opined that, endophytes from oilseed rape and tomato significantly reduced disease symptoms caused by *Verticillium dahliae* Kleb and *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.). Benhamou *et al.* (2000) suggested that, treatment with the endophytic bacterium, *Serratia plymuthica,* prior to *Pythium*

inoculation can reduce seedling disease development in cucumber (*Cucumis sativus*). Barka *et al.* (2002) demonstrated *in vitro* antagonism of endophytic *Pseudomonas* sp. on the growth of *Botrytis cinerea* infecting grapevine. Reiter *et al.* (2003) isolated endophytic strain of *Clavibacter michiganensis* from potato with biocontrol activities against *Erwinia carotovora.*

Li *et al.* (2003) isolated 55 bacterial strains from the tomato stem samples collected from different locations in China, of which, seven strains could inhibit the growth of *R. solanacearum.* Mohandas *et al.* (2004) observed that, treatment of banana roots with *P. fluorescens* could reduce 72 per cent colonization of *F. oxysporum f. sp. cubense.* Rubini *et al.* (2005) demonstrated the use of endophytic fungi and bacteria in the biocontrol of witches broom disease of cacao caused by *Crinipellis perniciosa.*

Rajendran *et al.* (2006) opined that, among biological control methods, endophytic bacteria are an alternative to systemic pesticides that can be more reliable and ecologically as well as economically sustainable. They also reported that, bacterial endophytes promote plant growth and improve host's capacity to withstand pathogen by inducing systemic resistance. Mathew (2008) emphasized the potentiality of endophytic *B. megaterium* from black pepper against *Phytophthora* disease of black pepper and vanilla and bacterial wilt of chilli under pot culture and field conditions. Aravind *et al.* (2009) reported three species of endophytic bacteria from roots and leaves of black pepper *viz. P. aeruginosa, P. putida* and *B. megaterium* which showed 70 per cent suppression of *P. capsici* infection in black pepper nursery. Balan (2009) studied the antagonistic effect of endophytic *Bacillus* sp. and *Pseudomonas* sp. against bacterial blight of anthurium. Mathew (2009) reported the antagonistic effect of *P. stutzeri* against bacterial wilt of ginger and chilli and soft rot of ginger. According to Kurian (2011), the endophytic bacteria *P. putida, B. subtilis, P. plecoglossicida* and *P. aeruginosa* were found effective against

Phytophthora pod rot of cocoa. Purnawati *et al.* (2014) noticed the antagonistic activity of the bacterial endophytes of tomato against *R. solanacearum*.

Amaresan *et al.* (2012) isolated 82 endophytic bacteria of tomato and chilli from different locations of tropical Islands of Andaman and Nicobar, India and *in vitro* screening, 16 bacterial isolates were found effective against *R.solanacearum*. Feng *et al.* (2013) found that, the endophytic bacterial communities present in bacterial wilt resistant tomato varieties at different growth stages were significantly higher than those in susceptible plants. Seven endophytic bacterial genetic groups identified in resistant cultivars belonged to *Spingomonas yanoikuyae, P. pseudoalcaligenes, Serratia marcescens, B. megaterium, Paenibacillus polymyxa, B. pumilus* and *B. cereus* and tested for their antagonistic efficiency against *R. solanacearum*. Purnawati *et al.* (2014) also noticed the antagonistic activity of the bacterial endophytes of tomato against *R. solanacearum*.

The role of endophytic bacteria in plant growth promotion was established by Sturz *et al.* (1998) and of the 25 isolates of endophytic bacteria tested in potato plant bioassays, 63 per cent increased shoot height, 66 per cent increased shoot fresh weight, and 55 per cent increased root fresh weight. According to Fu *et al.* (1999), the endophytic bacterial isolate (Strain 73a) of cotton promoted shoot growth by 19.15 per cent. Similar observations were made by Nejad and Johnson (2000) who observed that, the endophytic isolates from oilseed rape caused significant increase in shoot fresh weight compared to control and significantly improved seed germination, seedling height and plant growth when used as seed treatment. Bacon and Hinton (2002) recorded 70 per cent increase over control in root and shoot growth of corn and beans by application of endophytic *B. mojavensis*.

Although the interaction between endophytes and host plants has not been fully understood, many fungal and bacterial endophytes were reported to promote plant growth and the mechanism attributed includes nitrogen fixation, production of growth-promoting substances and increased resistance to pathogens and parasites

(Muthukumarasamy *et al.*, 2002). Barka *et al.* (2002) observed faster growth of grape plants with more secondary roots and leaf hairs when co-cultured with an endophytic bacterium *Pseudomonas* sp. In another study, cabbage dipped in *B. subtilis* suspension for 24 h before sowing recorded 91.20-138.04 per cent increase in fresh weight compared to the control (Hong *et al.*, 2004). Forchetti *et al.* (2010) supported the fact that, endophytic bacteria enhanced growth of sunflower seedlings even under water stress conditions.

2.7.2. Endophytic fungi

Gasoni and de Gurfinkel (1997) reported the suppression of *Rhizoctonia solani* by the endophytic fungus *Cladorrhinum foecundissimum* in cotton plants. According to Redman *et al.* (1999), a non-pathogenic mutant of *C. magna* (path-1) protected watermelon and cucumber seedlings from anthracnose disease caused by wild-type *C. magna*. Samuels *et al.* (2000) reported endophytic *T. stromaticum* against cacao witches' broom pathogen. Arnold *et al.* (2003) found that, inoculation of endophyte-free leaves with endophytes isolated from naturally infected asymptomatic hosts, significantly reduced both leaf necrosis and mortality of cocoa seedlings inoculated with *Phytophthora* sp. Holmes *et al.* (2004) also showed the ability of *T. ovalisporum* to colonize within the intact plumule of cocoa seedling conferring resistance to *Moniliophthora* spp. Tondje *et al.* (2006) reported the efficacy of endophytic fungus *Genicolosporium* strain against *P. megakarya* sporulation in cocoa.

Sobowale *et al.* (2007) observed the efficacy of endophytic *T. pseudokoningii* and *T. harzianum* against *F. verticilloides* in maize and also stated that, reisolation of any of the Trichoderma species from different points within maize stem other than point of inoculation would be suggestive of their endophytic ability. Mejia *et al.* (2008) reported endophytic *Trichoderma* spp. as biocontrol agent against *Moniliophthora roreri* in cacao. Bailey *et al.* (2008) also noticed antagonistic activity

of endophytic *T. harzianum*, *T. hamatum* and *T. asperellum* against *Moniliophthora roreri* of cacao.

Kim *et al.* (2007) found that, among the endophytic fungi isolated from healthy tissues of vegetable plants, *F. oxysporum* was most effective against *P. ultimum*, *P. infestans* and *P. capsici in vivo*. Mathew (2008) observed the antagonistic effect of endophytic *T. viride* and *T. pseudokoningii* isolated from black pepper against bacterial wilt of chilli and *Phytophthora* rot of black pepper and vanilla under pot and field condition. Mejia *et al.* (2008) assessed the effects of three endophytic fungi (*C. gloeosporioides*, *Clonostachys rosea* and *Botryosphaeria ribis*) on cocoa pod rot due to *Moniliophthora roreri* and *Phytophthora* spp. and observed that, the treatment with *C. gloeosporioides* and *C. rosea* significantly reduced the disease incidence.

Hanada *et al.* (2008) isolated a new species of *Trichoderma martiale* as an endophyte from the sapwood of *Theobroma cacao* in Brazil and they reported that, the selected *Trichoderma* isolate was found to have good endophytic ability, as well as antagonistic activities such as mycoparasitism, antibiosis, and induced resistance, which could be exploited for various biocontrol strategies of cocoa diseases. M'pika *et al.* (2009) observed that, of 139 *Trichoderma* isolates tested, 26 *Trichoderma* isolates could reduce more than 50 per cent cocoa pod rot incidence due to *P. palmivora*. Andrade-Linares *et al.* (2011) obtained 51 endophytic fungal isolates from tomato roots, of which, 20 isolates belonged to *Fusarium* species and only three isolates showed antagonistic property.

Bae *et al.* (2009) reported that, endophytic colonization of *T. hamatum* in cocoa seedlings resulted in an increase in biomass weight leading to delay in onset of drought response. Dias *et al.* (2009) observed the ability of 20 endophytes isolated from strawberry to enhance the root number, length and dry weight and also the leaf number, petiole length and dry weight of the aerial portion. Kurian (2011) also

reported that, the endophytic isolates augmented the growth parameters in cacao seedlings especially the seedling height.

2.7.3. Endophytic actinomycetes

The use of endophytic actinomycetes as bioagents of soil borne root diseases is of interest through their ability to colonize healthy plant tissues and produce antibiotics *in situ* (Cao *et al.*, 2005). Verma *et al.* (2009) observed that, endophytic actinomycetes have attracted attention in the search for novel bioactive natural compounds that can be used to design new drugs replacing those against which pathogens have acquired resistance.

Actinomycetes secrete hydrolytic enzymes such as cellulases, hemi cellulases, chitinases, amylases and glucanases which degrade the cell wall of pathogen (Getha and Vikineswary, 2002). Taechowisan *et al.* (2003) screened more than 300 isolates of endophytic actinomycetes for their potential to produce chitinase and found that *S. aureofaciens* was the most effective producer of chitinase and the crude purified enzyme had potential for cell wall lysis of many phytopathogenic fungi. Tan *et al.* (2006) reported that, endophytic actinomycetes isolated from stem and roots of different cultivars of tomato varied in their antagonistic potential against *R. solanacearum* under *in vitro* condition.

According to El-Tarabily *et al.* (2009), endophytic actinomycetes *viz. Actinoplanes companulatus*, *Micromonospora chalcea* and *S. spirales* produced glucanases and protected cucumber plants from the infection of *P. aphanidermatum* under greenhouse condition. Ning *et al.* (2010) opined that, *S. longisporflavus* showed high inhibition efficiency on *Sclerotinia sclerotiorum* by the production of chitinases. El-Tarabily *et al.* (2010) observed that, the success of the endophytic actinomycetes, *S. spiralis* over *Actinoplanes companulatus* in the control of *P. aphanidermatum* of cucumber under field condition was due to its ability to produce higher levels of glucanases. Nimnoi *et al.* (2010) noticed protease activity in 10

endophytic actinomycetes isolated from healthy shoots and roots of *Aquilaria crassna* (eagle wood).

Moura *et al.* (1998) observed that, endophytic actinomycetes isolated from the root tissues of tomato showed cent per cent control of the pathogen. Moura and de-Romero (1999) also noted that, endophytic actinomycetes isolated from various hosts showed a high inhibitory activity against *R. solanacearum*. Sreeja (2011) studied the efficacy of five isolates of endophytic actinomycetes of tomato collected from Thrissur and Palakkad areas of Kerala against bacterial wilt disease and indicated that, Ozhalapathy endophytic isolate, *S. thermodiastaticus* was the most efficient one in the management of bacterial wilt as well as in plant growth promotion in tomato.

Franco *et al.* (2006) noted that, seed treatment with endophytic actinomycetes was beneficial for cereal crops through enhanced germination and plant growth. El-Tarabily *et al.* (2009) evaluated the potentiality of three endophytic actinomycetes viz. *Actinoplanes companulatus*, *Micromonospora chalcea* and *S. spirales* in the plant growth promoting activity of cucumber and observed significant increase in root and shoot production and enhanced level of growth promotion when they were combined together.

2.8. Mode of action of endophytes

Endophytic biocontrol agents can be divided into two groups: (i) strains that extensively colonize the internal plant tissues and suppress invading pathogens by occupying niche, antibiosis, or both, and (ii) strains that primarily colonize the root cortex where they stimulate general plant defense/resistance mechanisms.

Cook and Baker (1983) suggested different mechanisms including production of antifungal compounds, siderophore, nutrient competition, niche exclusion and induction of systemic resistance by endophytic microbes in controlling *Fusarium* wilt of different crops. According to Backman *et al.* (1997), the effectiveness of

endophytes as biocontrol agents is dependent on many factors like host specificity, population dynamics, pattern of host colonization, ability to move within host tissues, and ability to induce systemic resistance. Certain endophytic bacteria trigger a phenomenon known as induced systemic resistance (ISR), which is phenotypically similar to systemic-acquired resistance (SAR). SAR develops when plants successfully activate their defense mechanism in response to primary infection by a pathogen especially when the pathogen induces a hypersensitive reaction. ISR is effective against different types of pathogens but differs from SAR in that the inducing bacterium does not cause visible symptoms on the host plant (Van Loon *et al.*, 1998). Bacterial endophytes prevent disease development through endophyte mediated synthesis of structural compounds and fungal toxic metabolites which may lead to certain forms of systemic acquired resistance (Sturz *et al.*, 2000).

Endophytic bacteria inhibit the growth of pathogen by production of antimicrobial compounds (Leyns *et al.*, 1990), siderophore (Kloepper *et al.*, 1980), or by nutrient competition (Lockwood, 1990). They are capable of inducing systemic resistance and other defence responses (Vanpeer *et al.*, 1991) along with deposition of structural barriers in cell wall (Benhamou *et al.*, 1998). Ongena *et al.* (2007) found that the fengycin and iturin lipopeptides are responsible for most antifungal activity of *B. subtilis* in *in vitro* assays against *F. oxysporum*. In addition to antagonistic activity, these cyclic lipopeptides are known to trigger induced systemic resistance. Production of salicylic acid by the endophytic isolates also plays a significant role in imparting resistance. De Meyer and Hofte (1997) observed that, rhizobacterium *P. aeruginosa* produced salicylic acid in beans, thereby inducing resistance to leaf infection by *B. cinerea*. Similarly, induced systemic resistance has been reported in different crops by the *in planta* accumulation of salicylic acid (De Meyer *et al.*, 1999; Vijayaraghavan, 2007). Nandhini *et al.* (2012) proved that, endophytic bacteria from tomato, belonged to *Bacillus*, *Pseudomonas*, *Klebsiella* and *Citrobacter*, produced salicylic acid *in vitro*.

According to Bloemberg and Lutenberg (2001), the widely recognized mechanisms of biocontrol mediated by endophytic bacteria are competition for an ecological niche or a substrate, production of inhibitory allelochemicals, and induction of systemic resistance in host plants to a broad spectrum of pathogens and/or abiotic stresses.

Production of volatile inhibitory substances by endophytes was studied by Nejad and Johnson (2000). They found that, most of the endophytic isolates from oil seed rape were HCN negative but the isolates produced other volatile metabolites which had fungal inhibitory action. Hence they concluded that, the endophytes are producing antifungal volatiles other than HCN. Volatile substances such as 2-3 butanediol and aceotin produced by bacteria have been reported to be responsible for plant growth promotion (Ryu *et al.* 2003). The production of hydrolytic enzymes by endophytic microorganisms is another determinant of different biocontrol mechanisms (Connelly *et al.*, 2004). Endophytes from potato showed antagonistic activity against fungi and also inhibited bacterial pathogens belonging to the genera *Erwinia* and *Xanthomonas* by producing antibiotics and siderophores *in vitro* (Sessitsch *et al.*, 2004). Rini and Sulochana (2007) reported that, *Trichoderma* produced both volatile and nonvolatile substances that suppressed the growth of *R. solani* and *F. oxysporum*.

Rajendran *et al.* (2006) and Azevedo and Araujo (2007) opined that, bacterial endophytes promote plant growth and improve host capacity to withstand pathogen attack by competition, antibiosis and induced systemic resistance. Of the 103 endophytic bacterial strains isolated from cotton, two strains of *Bacillus* spp. suppressed the damping off disease by inhibiting the mycelial growth of *R. solani* (Rajendran and Samiyappan, 2008).

2.9. Induced systemic resistance

Plant immunization for resistance against a wide variety of phytopathogens is an effective strategy for plant disease management. According to Vance *et al.* (1980), the biochemical mechanism involved in the plant disease resistance is a complex phenomenon. Induced lignification has been proposed as a mechanism of disease resistance against invasion of the pathogens and the enzymes phenylalanine ammonia lyase (PAL) and peroxidase (PO) play important role in the lignin biosynthesis. Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated (Kuc, 1982).

White and Cole (1985) opined that, endophytes are important in epidemiology because endophytic associations lead to the enhancement of resistance in plants. High levels of phenol synthesis, rapid lignifications and localized necrotization contribute resistance in plants against pathogen. Endophytes are capable of inducing systemic resistance and other defence responses in the host plant including the production of phytoalexins (Vanpeer *et al.*, 1991), accumulation of pathogenesis-related proteins (Zdor and Anderson, 1992), deposition of structural barriers in the cell wall (Benhamou *et al.*, 1996), and thus offer protection to host plant from the attack of wide variety of pathogens. Duijff *et al.* (1997) reported that, colonization of epidermal or hypodermal cells in tomato by endophytic *P. fluorescens* led to the thickening of cortical cell walls thus resulting in induction of resistance to Fusarium wilt in tomato.

Viswanathan and Samiyappan (1999) revealed the ability of endophytic *P. fluorescens* isolated from stalk tissues of sugarcane in inducing systemic resistance against red rot disease. Benhamou *et al.* (2000) studied the potential of the endophytic bacterium *Serratia plymuthica* in stimulating defence reactions in cucumber. Histological investigations of root samples of cucumber revealed differences in the plant defence reactions between bacterized and non-bacterized

plants which resulted in restriction of fungal colonization to the outermost root tissues of bacterized seedlings.

PGPR mediated stimulation of defence related biochemical compounds have been reported by earlier workers (Chen *et al.,* 2000; Maurhofer *et al.*, 1994; M'Piga *et al.*, 1997; Zdor and Anderson, 1992). Nandakumar *et al.* (2001) reported that, rice cv. IR-50 treated with *P. fluorescens* induced, systemic resistance in rice plants due to the production of chitinase and peroxidase. Adhikari *et al.* (2001) evaluated endophytic bacteria obtained from rice and found effective for plant growth promotion and biological control of seedling disease of rice. Induction of plant defence mechanisms has been suggested as an important benefit rendered by endophytic microbes (Bargabus *et al.*, 2002). *Bacillus* strains produced several cyclic peptides, amino-polyols and amino-glycosides, which are having significant effect on ISR development (Yu *et al.*, 2002). Endophyte-triggered ISR fortifies plant cell wall strength and alters host physiology and metabolic responses, leading to an enhanced synthesis of plant defense chemicals upon pathogen invasion and/or abiotic stress factors (Nowak and Shulaev, 2003).

Rajendran *et al.* (2006) opined that, bacterial endophytes of cotton promote plant growth and improve host's capacity to withstand the attack by *Xanthomonas axonopodis* pv. *malvacearum* by inducing systemic resistance. Uppala (2007) observed increased activity of defense related enzymes in amaranth by the application of endophytic *Bacillus* sp. and *Pseudomonas* sp. for the management of leaf blight disease. The ability of four *Bacillus* sp. isolated from vegetable crops to colonize in cocoa seedlings and reduction in the severity of black pod rot was evaluated by Melnick *et al.* (2008). Kurian (2011) studied the effect of endophytes in cocoa seedlings and observed enhanced production of phenols, oxidative enzymes and β-1,3-glucanase, thus inducing systemic resistance against *Phytophthora* pod rot of cocoa.

2.9.1. Phenols

Enhanced formation of defence related compounds in plants as a result of endophyte treatment was studied by many workers. Malinowski *et al.* (1998) reported that, total phenolic concentration was 20 per cent greater in shoots of endophyte infected plants than uninfected plants.

The plant response induced in bacterized plants, after challenge inoculation with a pathogen resulted in the formation of structural barriers, such as thickened cell wall papillae due to the deposition of callose and the accumulation of phenolic compounds at the site of pathogen attack (Benhamou *et al.*, 1998). Glucosides, lipids, and phenolics were detected in the wall appositions formed in cucumber roots when treated with endophytic bacterium *Serratia plymuthica* offering protection against *P. ultimum* (Benhamou *et al.*, 2000).

The high toxicity of total and OD phenols and their role in plant resistance was reported earlier by Mahadevan (1966 & 1970). He reported that, the increased rate of oxidation of phenolics to more toxic compounds like quinones by the oxidative enzymes like polyphenol oxidase and peroxidase. Orthodihydroxy phenolic compounds such as caffeic acid, and chlorogenic acid, and orthoquinones and tannins were shown to strongly inhibit the activities of extracellular enzymes produced by microorganisms.

Phenolic compounds enhanced the mechanical strength of the host cell walls in cotton and also inhibited the invading *Xam* (Rajendran *et al.*, 2006). Ganley *et al.* (2008) also reported a relatively higher quantity of lignification (30 - 100% over control) in the bacterized roots compared to the plants untreated which resulted in significant root rot suppression. Tomiyama (1963) opined that, mono and dihydric phenols increased in host tissues invaded by parasites as a part of resistance mechanism.

Khatun *et al.* (2009) estimated the total phenol content along with the oxidizing enzymes, peroxidase and polyphenol oxidase, in diseased leaves of rose caused by *Alternaria tenuis* at different progression periods of infection and found that, there was increased activity of oxidizing enzymes in host plants in response to infection, which was found to be coupled with an increased phenol content in diseased leaf tissue as compared to the healthy ones. This clearly reveals that, the elicitation of the enzymes in response to infection is directly correlated with the resistance of the plant.

Higher accumulation of phenolics by prior application of *P. fluorescens* challenged with the pathogen has been reported in various crops (Meena *et al.*, 2000; Singh *et al.*, 2003; Vivekananthan *et al.*, 2004). Jain *et al.* (2012) observed higher accumulation of phenols in pea leaves treated with microbial consortia compared with untreated plants challenged or unchallenged with *Sclerotinia sclerotiorum*. Total phenols and ortho-dihydroxy phenols from healthy and *S. rolfsii* inoculated groundnut plants were estimated at different stages of disease development after 10 days of inoculation and observed that, the increased levels of phenols, OD phenols and the enzymes like PAL, PPO and PO formed an important part in the resistance mechanism of the groundnut plants against infection with *S.rolfsii* (Saraswathi and Reddy, 2012).

2.9.2. Defense enzymes

Other than the phenolic compounds, increase in the level of defence related enzymes has also been reported by various workers with treatment of endophytes. Rodriguez *et al.* (2001) studied the roots of tomato plants cultivated using mycorrhizal inoculant-coated seeds, in order to evaluate the induction dynamics of defense mechanisms in the tomato-*Glomus clarum* and tomato-*Glomus fasciculatum* interaction through peroxidase and polyphenol oxidase behavior and found that, both enzymes kept low activity levels at early symbiosis stages; thereinafter enzymatic

activities increased in colonized roots reaching a maximum, which was higher in *G. clarum*-inoculated plants, and later these activities declined.

Higher levels of peroxidase have been correlated with enhanced ISR in several plants treated with endophytes (Kandan *et al.*, 2002; Ramamoorthy *et al.*, 2002a). Peroxidase is a phenol oxidizing enzyme which oxidizes phenols to form quinones and also generate hydrogen peroxide. It is a key enzyme in the biosynthesis of lignin (Bruce and West, 1989; Brisson *et al.*, 1994). Anita *et al.* (2004) carried out an *in vitro* experiment to study the induction of defense enzymes by *P. fluorescens* against challenge inoculation of *Meloidogyne incognita* in tomato and found that, activities of all the enzymes were significantly higher in bacterized tomato root tissues challenged with the nematode. Paul and Sarma (2005) proved that, increased levels of peroxidase, catalase, phenylalanine ammonia lyase and polyphenol oxidase were induced in leaves and roots of treated plants indicating the systemic protection offered to black pepper against foliar infection by the pathogen, *P. capsici*.

Rajendran *et al.* (2006) has also reported cotton plants treated with endophytic bacteria and challenged with *Xam* showed higher levels of PO as well as PPO. Uppala (2007) observed increased activity of PO, PPO, and PAL in amaranth by the application of endophytic *Bacillus* sp. and *Pseudomonas* sp. against *R. solani*. Daayf *et al.* (1997) reported that, PAL is a key enzyme in the production of phenols and phytoalexins. The study conducted by Vanitha *et al.* (2009) focused on the role of the defense-related enzymes, PAL and PPO in imparting resistance in tomato against *R.solanacearum*. The enzyme activities and total phenol content increased significantly in resistant cultivars on pathogen inoculation and the increase was not significant in susceptible and highly susceptible cultivars. PGPR induced peroxidase in response to pathogen attack (Anita and Samiyappan, 2012).

Sankari -Meena *et al.* (2012) observed increased level of oxidative enzymes in tomato roots by the application of *P. fluorescens* alone thereby reducing the root knot

nematode infestation which indicated the induction of systemic resistance by the bacterial formulation. Consortial formulation of biocontrol agents *viz. P. fluorescens* and *B. subtilis* recorded the highest enzymatic activity thereby inducing systemic resistance against *Meloidogyne incognita* in tomato by the accumulation of oxidative enzymes. Sivakumar *et al.* (2013) found elevated levels of defence related enzymes in the brinjal plants treated with *B. megaterium* when assayed against *R. solanacearum.*

2.9.3. PR proteins

The chitinases and β-1, 3 glucanases (which are classified under PR-3 and PR-2 groups of PR proteins respectively) are reported to be associated with greater resistance in plants induced by endophytes against pests and diseases (Maurhofer *et al.*, 1994; Van Loon, 1997). Biochemical or physiological changes in plants include induced accumulation of pathogenesis-related proteins (PR proteins) such as PR-1, PR-2, chitinases, and some peroxidises (Majeau *et al.*, 1990; M'Piga *et al.*, 1997).

De Meyer *et al.* (1998) reported that, systemic resistance in biocontrol of *B. cinerea* by *T. harzianum* T39 was induced by the accumulation of PR proteins. Meena *et al.* (2000) observed that, foliar application of plant growth-promoting rhizobacterium, *P. fluorescens* significantly controlled late leaf spot (*Cercosporidium personatum*) and rust (*Puccinia arachidis*) diseases of groundnut under greenhouse conditions. Groundnut plants, when sprayed with *P. fluorescens,* showed significant increase in activity of chitinase and β-1,3-glucanase one day after *P. fluorescens* treatment and reaching maximum at fourth day.

According to Ramamoorthy *et al.* (2002b), *P. fluorescens* was found to protect tomato plants from wilt disease caused by *Fol* by inducing defense proteins and chemicals. Phenolics and activities of PAL, PO and PPO increased in bacterized tomato root tissues at 24 h after challenge inoculation and the enzyme activities reached maximum at the 5^{th} day after challenge inoculation. Similarly,

β-1,3-glucanase, chitinase and thaumatin-like proteins (TLP) were induced to accumulate at higher levels at 3-5 days of challenge inoculation in bacterized plants. These results suggest that, induction of defense enzymes involved in phenylpropanoid pathway and accumulation of phenolics and PR-proteins might have contributed to restriction of invasion of *Fol* in tomato roots.

Soil application of the biocontrol agents (*P. fluorescens*, *T. viride* and *T. harzianum)* in combination with chitin, induced significant increase in the activities of PO, PPO, PAL, chitinase and β−1,3-glucanase in the *Ganoderma lucidum* infected palms (Karthikeyan *et al.*, 2006). Treatment with endophytic bacterial bioformulation increased the levels of phenols, defence related enzymes, chitinase and β-1, 3 glucanase in cotton plants which had been challenged with *R. solani* thus enhancing the resistance in cotton plants in addition to plant growth and antibiosis (Rajendran and Samiyappan, 2008).

The effect of biocontrol agent *B. subtilis* on the induction of PR proteins in tomato plants infected with *Fol* was investigated by Ramyabharathi *et al.* (2012). On spectrophotometric analysis, it was observed that, the maximum activity of these defense enzymes was observed in seedling dip, soil application and foliar spray with *B. subtilis* liquid formulation in tomato challenged with the pathogen. Activities of these defense enzymes reached maximum at the seventh day after inoculation of the pathogen. The results demonstrated that, both the defense enzymes might play a special role in pathogenesis during fungal infection. Increased β-1,3-glucanase activity on application of endophyte, *B. subtilis,* has been reported by Wilhelm *et al.* (1998) in chestnut against chestnut blight and Jayaraj *et al.* (2004) in rice against sheath blight.

2.10. Methods of inoculation

The methods by which endophytes are applied are similar to those used for applying other biological control agents. Successful colonization and maintenance of effective level of population of endophytic bacteria in the plant and in turn the level of disease control are mainly dependent on the method of delivery of these organisms into the host.

Quadt-Hallmann and Kloepper (1996) described some common procedures of inoculation of endophytes such as pouring bacterial suspension or mixing it with autoclaved soil or dipping the roots of seedlings in a bacterial suspension at the time of transplanting or seed coating with bacterial suspension before sowing.

Use of endophytes in biocontrol require introduction of endophytes into plant tissues in the quantity, site and life-history stages that effectively antagonise pathogens (Reinhold-Hurek and Hurek, 1998). When the endophytic bacteria are introduced into the seed, the bacteria survives and moves in the vegetative part and subsequently the propagative seeds will also have the introduced bacteria, thus minimizing the need for frequent application of bacterial strains (Ramamoorthy *et al.*, 2001). Sprays of spore suspensions have been used to introduce endophytes into beans and barley and introduction of endophytic bacteria into the host plant at an early growth stage avoids competition for colonization by other micro organisms, and allows attaining required levels of colonization, and thus avoiding need of subsequent inoculations (Boyle *et al.*, 2001).

Akbar (2002) noticed that, the seed treatment + soil drenching of *P. aeruginosa* was more effective than single application in reducing the wilt incidence in tomato. Manjula *et al.* (2002) compared the methods of inoculation of endophytes such as stab inoculation on stems, soaking seeds in bacterial suspensions, methyl cellulose seed coating, foliar spray, application of bacteria impregnated

granules in furrow, vacuum infiltration and pruned root dip, for the introduction of various endophytes into cotton. They found that, no single method of delivery was equally effective for various endophytes such as *Bacillus, Burkholderia, Cellulomonas, Clavibacter, Enterobacter, Phyllobacterium* and *Pseudomonas* spp. in cotton. Hence it was suggested that, different methods of inoculation are needed for efficient delivery of diverse strains.

Bhowmik *et al.* (2002) observed that, seed treatment with endophytic *Pseudomonas* sp. was the most effective in reducing infection by *X. malvacearum* in cotton. Manimala (2003) reported the maximum suppression of bacterial wilt in solanaceous vegetables with seed treatment + root dipping + soil application of bioagents. Hong *et al.* (2004) confirmed the colonization of endophytic *B. subtilis* in cabbage plants after inoculation by seed dipping, watering or leaf daubing. Combination of seed + soil + root dip + foliar spray of *P. fluorescens* was found highly effective against sheath blight of rice and also in increasing the durability of ISR in rice plants (Singh and Sinha, 2005). Sivakumar *et al.* (2011) observed effective disease suppression of bacterial wilt of brinjal by *B. megaterium* with the combined application of seed treatment + soil application + seedling root dip + foliar spray. According to Chakravarty and Kalita (2012), lowest per cent wilt incidence in brinjal was found with seed + root + soil treatment of *P. fluorescens*.

2.11. Consortium approach of bioagents

Recently, a greater thrust is given for the development of biological consortium since it consists of microbes with different biochemical and physiological capabilities, which permits interaction among themselves and will lead to the establishment of a stable and effective microbial community. It will further provide better management of diseases by way of synergistic effect and multiple mode of action. A search on literature revealed information on consortium approach of rhizospheric bioagents for the management of plant diseases. The effectiveness of

combined application of antagonists in disease reduction and growth promotion in crops have been reported by various workers.

Pierson and Weller (1994) tested fluorescent pseudomonads alone and in combinations for the ability to suppress take-all disease in wheat and it was observed that, certain combinations not only suppressed the disease but also enhanced yield in wheat. Duffy and Weller (1995) reported significant suppression of take-all of wheat when *Gaeumannomyces graminis* var. *graminis* isolated from rice was applied in combination with fluorescent *Pseudomonas* spp. than either treatment used alone. *T. koningii* combined with either *P. chlororaphis* or *P. fluorescens* provided greater suppression of take-all of wheat than *T. koningii* alone (Duffy *et al.*, 1996). Spraying a mixture of *P. fluorescens* and *Erwinia herbicola* was found to be effective for the suppression of fire blight of pear (Nuclo *et al.*, 1998).

Raupach and Kloepper (1998) reported that, combined application of antagonistic strains *viz. Bacillus pumilus*, *B. subtilis*, and *Curtobacterium flaccumfaciens* enhanced growth promotion and disease reduction in cucumber, when compared with the strains tested singly. Sarma and Anandraj (1998) suggested the consortial approach for disease management in plantation and spice crops. Sarma *et al.* (2000) observed maximum disease suppression in black pepper, cardamom and ginger by the combination of *T. harzianum* (IISR 1369) and *P. fluorescens* (IISR-6).

Jisha *et al.* (2002) studied mutual compatibility of fungal and bacterial antagonists *viz. T. harzianum* and fluorescent Pseudomonads and developed an efficient consortium for the management of foot rot of black pepper caused by *P. capsici*. According to Guetsky *et al.* (2002), a combination of bio-control agents with different mechanisms of disease control will have an additive effect in enhancing disease control and biometric characters compared to their individual application. Jetiyanon and Kloepper (2002) also proposed consortial application of different biocontrol agents for improved and stable control against a complex of

diseases. Anandaraj and Sarma (2003) evaluated the consortial effect of different combinations of five bacterial strains which had been proved efficient in suppressing *P. capsici* and growth promotion in black pepper and was found highly effective when used in combination. Mathew (2008) observed that, consortial application of *T. harzianum* with *P. fluorescens* or *B. megaterium, T. harzianum* + *T. viride* provided better management of Phytophthora rot of black pepper and vanilla, and bacterial wilt of chilli. Mathew (2009) also reported that, combined application of *T. harzianum* + *P. fluorescens, T. harzianum* + *T. virens and P. fluorescens* + *B. subtilis* were effective in suppressing rhizome rot and bacterial wilt in ginger and chilli and also observed drastic effect on plant growth and yield compared to individual applications. Many researchers have also observed the consortial effect of various biocontrol organisms such as *Trichoderma*, *Pseudomonas*, *Bacillus* spp. etc in chickpea, tomato, chilli, Arabidopsis and pigeon pea (Rudresh *et al.*, 2005; Kannan and Surender, 2009; Srivastava *et al.*, 2010).

Lucas *et al.* (2009) compared the individual and consortial effects of *P. fluorescens* and *Chryseobacterium balustinum* for the management of *Magnaporte grisea* in rice and observed that, the combination of both strains was the most effective treatment compared to individual strain, with 50 per cent protection against blast disease and also increased productivity and quality of rice. Singh *et al.* (2013) conducted a study to examine efficacy of a rhizospheric microbial consortium comprising of a fluorescent pseudomonad (PHU094), *Trichoderma* (THU0816) and *Rhizobium* (RL091) strain on activation of physiological defense responses in chickpea against the collar rot pathogen, *S. rolfsii,* and the studies revealed maximum activities of defense enzymes and accumulation of total phenol contents in the triple microbial consortium treated plants challenged with the pathogen, compared to the single microbe and dual microbial consortium. Singh *et al.* (2014) also reported that, the rhizosphere competent microbial consortium consisting of three compatible microbes, *viz. P. aeruginosa, T. harzianum* and *Mesorhizobium* sp. mediated, rapid

changes in phenolic profiles in chickpea during *S. rolfsii* infection and the consortial application led to higher growth in chick-pea particularly in the triple microbe combination compared to their individual treatments and control.

Search on literature revealed that, the information on the consortial effect of endophytes are meagre and scanty and the available reports are included in this chapter. Aravind *et al.* (2009) reported that, the consortial application of endophytic bacteria from roots and leaves of black pepper consisted of *P. aeruginosa, P. putida* and *B. megaterium* showed 70 per cent suppression of *P. capsici* infection in black pepper nursery. Muthukumar *et al.* (2011) evaluated the individual and combined application of talc-based formulation of *T. viride* and endophytic *P. fluorescens* for the control of *P. aphanidermatum* in chilli and observed maximum induction of defense-related enzymes, PR-proteins and accumulation of phenolics in consortial application compared with the individual ones.

Sundaramoorthy and Balabaskar (2012) made an attempt to develop an effective ecofriendly strategy to manage early blight disease of tomato using endophytic and plant growth promoting rhizobacteria, *B. subtilis* and *P. fluorescens* and observed significant reduction in early blight incidence under greenhouse conditions with the combined application of *B. subtilis* + *P. fluorescens* suggesting the synergistic effect of the consortium.

Materials and Methods

3. MATERIALS AND METHODS

Investigation on "Enhancement of resistance to bacterial wilt in tomato by endophytic microbial communities" was conducted in the Department of Plant Pathology during the period 2011-2014. Biochemical and molecular works were carried out in the Department of Agricultural Microbiology, and Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara.

3.1. STANDARDIZATION OF DILUTION FACTOR FOR THE ISOLATION OF ENDOPHYTIC MICROORGANISMS

A preliminary experiment was carried out for the standardization of dilution factor for the isolation of endophytic microorganisms. The root and stem portions of tomato plants collected from research plot of Department of Olericulture, Vellanikkara were used for the study.

Healthy tomato plants adjacent to wilted ones were uprooted, brought to the laboratory, and washed under running tap water to remove the soil particles adhering to the root. The root portion 5 cm below the soil line and the stem portion 10 cm above the soil line were taken for the isolation. The skin of the stem was peeled off and the root skin scraped off to remove the external contaminants and these were cut into bits of 1 cm length.

3.1.1. Isolation of endophytic bacteria

Endophytic bacteria were isolated from root and stem samples as suggested by McInroy and Kloepper (1995). The stem and root bits of one gram were weighed separately. Stem samples were disinfested with 20 per cent hydrogen peroxide and root samples with 1.05 per cent sodium hypochlorite for 10 min and rinsed four times with sterile 0.02 M tris phosphate buffer (pH 7). An aliquot of 1 ml of the final buffer wash was transferred to sterile Petri plate to which nutrient agar (NA) was added and it served as sterility check. Each sample was triturated in 9 ml of

final buffer wash using a sterile pestle and mortar and dilutions were prepared up to 10^{-6} from this triturate. One ml from each dilution was pipetted into sterile Petri plates and 15 ml each of molten and cooled NA and King's B media (Appendix I) were poured separately and the plates were incubated at room temperature (28 ± 2^0C) for 48 h.

3.1.2. Isolation of endophytic fungi

The isolation of endophytic fungi from stem and root samples were carried out according to Haiyan *et al.* (2005). One gram each of stem and root samples were surface sterilized separately by sequentially treating with 0.5 per cent sodium hypochlorite and 70 per cent ethanol for 2 min, and rinsed with two changes of sterile water followed by two changes of sterile 0.02 M tris phosphate buffer. Sterility checks were kept on Martin's Rose Bengal Agar (MRBA) mediated plates. The samples were then triturated in 9 ml of final buffer wash and serial dilutions were prepared up to 10^{-3}. From each dilution, 1 ml was poured into sterile Petri plates and 15 ml each of molten and cooled MRBA and Trichoderma Selective Medium (TSM) (Appendix I) were poured separately and the plates were incubated at room temperature for 72 h.

3.1.3. Isolation of endophytic actinomycetes

Endophytic actinomycetes were isolated from root and stem samples adopting the protocol of Tan *et al.* (2006). The stem and root bits of one gram were weighed separately and the root samples were surface sterilized with 70 per cent ethanol for 30 sec, followed by 1.05 per cent sodium hypochlorite for 5 min and the stem bits with 20 per cent hydrogen peroxide for 10 min. The samples were then soaked in 10 per cent sodium bicarbonate solution for 10 min and, rinsed four times in 0.02 M sterile phosphate buffer. Sterility checks were maintained on Kenknight's Agar medium (KAM) (Appendix I). Each sample was ground in 9 ml of final buffer wash and dilutions up to 10^{-3} were prepared. One ml from each dilution was pipetted into

Petri plates to which 15 ml of molten and cooled KAM was poured and the plates were incubated at 28 ± 2^{0}C for seven days.

Microbial population in each dilution were recorded and the number of colonies in each sample was calculated using the following equation.

$$\text{Number of cfu/g sample} = \frac{\text{Number of colonies x dilution factor}}{\text{Volume plated (ml)}}$$

3.2. COLLECTION OF SAMPLES

A purposive survey was conducted for the collection of the samples. Healthy tomato plants were collected from 16 different locations representing north, central and south Kerala namely Padanakkad (Kasaragod District), Panniyur (Kannur), Malappuram and Tavanur (Malappuram), Ozhalapathy and Eruthempathy (Palakkad), Vellanikkara, Mannuthy and Cherumkuzhy (Thrissur), Kalamassery (Ernakulam), Kumarakom (Kottayam), Alappuzha and Kayamkulam (Alappuzha), and Kallambalam, Vellayani and Amburi (Thiruvananthapuram).

3.2.1. Isolation of endophytes from the samples of different locations

The endophytic microorganisms *viz.* bacteria, fungi and actinomycetes were isolated from the samples collected from different locations using the standardized dilution factors for each organism and as per the protocols mentioned earlier. The predominant microbial colonies were selected, purified and the pure cultures were maintained on potato dextrose agar (PDA) slants (Appendix I) for further studies. The isolates were numbered representing the place of collection, plant part from which it was isolated, the type of organism and the Arabic numerals in serial order.

3.3. ISOLATION OF BACTERIAL WILT PATHOGEN

The bacterial wilt pathogen of tomato was isolated from the infected plants as suggested by Kelman (1954). The wilted plants collected from the field, washed

under running tap water and then subjected to ooze test. The stem portion of the infected plant was cut into small segments of 1 cm and the surface sterilized bits were placed on a glass slide with a drop of sterile water, and teased apart. A loopful of the turbid suspension was streaked on triphenyl tetrazolium chloride (TZC) medium (Appendix I) and the plates were incubated at room temperature for 48 h. Typical white colonies with pinkish centre were selected, purified, and maintained on NA slants and in sterile water and kept in refrigerated conditions for further studies.

3.4. *IN VITRO* EVALUATION OF ISOLATED ENDOPHYTES FOR ANTAGONISTIC ACTIVITY AGAINST *R. SOLANACEARUM*

The isolated endophytes were tested for their antagonistic reaction against *R. solanacearum* adopting simultaneous antagonism method. Two day old bacterial, five day old fungal and seven day old actinomycetes cultures were used for *in vitro* evaluation. The culture media which favour the growth of both antagonists and the pathogen *viz.* PDA for fungi and actinomycetes and NA for bacteria were used.

3.4.1. *In vitro* evaluation of endophytic bacteria against *R. solanacearum*

A total of 79 bacterial endophytes obtained from different locations were screened against *R. solanacearum* by point inoculation technique. For the preliminary evaluation, the bacterial suspension of the pathogen was prepared by adjusting the concentration to 10^8 cfu ml^{-1} and 0.1 ml of the suspension was spread on NA medium using a glass spreader. A loopful of four different antagonistic organisms were spotted on the bacterial lawn at four equidistant points of 2 cm from the plate periphery. Plates were incubated at 28 ± 2^0C for 48-72 h and observed for the inhibition of the pathogen. The organisms that showed antagonistic reaction were selected for further studies.

Bacterial isolates, which showed antagonistic reactions in the initial screening, were tested individually by agar well method. Bacterial lawn was prepared

on nutrient agar mediated plates. Eight mm sized wells were made at the centre of the plates using a sterile cork borer and these wells were filled with 50 µl of 48 h old endophytic bacterial suspension. Plates with pathogen alone served as control. Petri dishes were incubated for 48 h with three replications for each antagonist. The diameter of inhibition zone was measured and the per cent inhibition was calculated using the formula suggested by Vincent (1927).

$$\text{Per cent Inhibition} = \frac{C - T}{C} \times 100$$

Where C = Radial growth of pathogen in control (cm)

T = Radial growth of pathogen in treatment (cm)

3.4.2. *In vitro* evaluation of fungal endophytes against *R. solanacearum*

The isolated 68 fungal endophytes were screened against *R. solanacearum*. In the initial screening, the pathogen was spread on PDA mediated Petri plates and four different candidate organisms were placed simultaneously at four corners of the culture plates at equidistant points. These plates were incubated at 28 ± 2^{0}C for five days. Those organisms showing antagonistic reactions were selected for further studies.

The antagonistic fungi selected from preliminary screening were then tested individually for its antagonistic property. Eight mm mycelial discs of selected fungi were placed individually at the centre of the PDA medium seeded with the pathogen and the plates were incubated at room temperature for five days. Plates with endophytic fungi alone served as control. Observations were taken till full growth in control plates, and per cent inhibition of the pathogen was calculated.

3.4.3. *In vitro* evaluation of endophytic actinomycetes against *R. solanacearum*

Seven endophytic actinomycetes were tested against *R. solanacearum.* For preliminary screening, four mm discs of the antagonists were placed at four corners

on the bacterial lawn at equidistance and incubated for seven days at room temperature.

The promising antagonists were selected and tested individually using agar well method in triplicates. The diameter of the inhibition zone was measured and the per cent inhibition of the pathogen was calculated.

3.5. SCREENING OF SELECTED ENDOPHYTES FOR ANTAGONISTIC POTENTIAL UNDER *IN PLANTA* CONDITION

The promising endophytes selected from *in vitro* studies were evaluated for their antagonistic activity using a highly susceptible variety, PKM-1 under *in planta* condition. Nursery was raised in earthern pots containing sterilized potting mixture consisting soil, sand, and cow dung @ 2:1:1. The potting mixture was sterilized using 4 per cent formaldehyde solution and covered with a polythene sheet for 10 days. It was then kept open for two days with intermittent raking to remove the traces of formaldehyde fumes. This potting mixture was used for raising the nursery and for growing tomato seedlings in polybags.

For the preparation of antagonist suspensions, the selected endophytic organisms were grown in different liquid media like potato dextrose broth (PDB) for fungi, nutrient broth (NB) for bacteria, and Kenknight broth for actinomycetes and incubated for 7, 2, and 14 days respectively. This was followed in all selected experiments, unless otherwise mentioned.

Twenty five day old seedlings were planted in black polybags of size 30 cm x 20 cm containing sterilized potting mixture. The antagonists were applied to the soil at the time of planting @ 30ml/plant having concentrations of 10^6 spores ml^{-1} for fungi, 10^8 cfu ml^{-1} for bacteria and 10^5 cfu ml^{-1} for actinomycetes. Challenge inoculation of the pathogen was done with fresh bacterial ooze suspension having concentration of OD_{600} = 0.3 @ 10 ml/plant by soil drenching with wounding at 30

days after planting. This procedure was adopted in all other *in planta*/ pot culture experiments. Three replications with 12 plants in each were maintained for the experiments. Wilt incidence was recorded 10 days after inoculation. Per cent wilt incidence was calculated using the following formula.

$$\text{Per cent wilt incidence} = \frac{\text{Number of plants wilted}}{\text{Total number of plants observed}} \times 100$$

3.6. STUDY OF MUTUAL COMPATIBILITY OF PROMISING ANTAGONISTS

Promising antagonists including fungi, bacteria and actinomycetes selected from the above study were tested for their mutual compatibility *in vitro* using PDA and selective media of the respective organisms.

3.6.1. Compatibility between fungal endophytes

The interaction between the fungal endophytes was studied by dual culture technique. Eight mm mycelial discs of seven day old cultures of two fungi were placed at opposite ends in Petri dishes with PDA. Plates were kept in triplicates for each combination and incubated at room temperature for five days to observe the compatibility reaction.

3.6.2. Compatibility between bacterial endophytes

The selected bacterial endophytes were tested for compatibility by cross streak method. Two different bacterial isolates were streaked vertically and horizontally on NA mediated plates. The plates were incubated for 48 h at room temperature and observed for lysis at the juncture of the streaks.

3.6.3. Compatibility between actinomycetes

The compatibility between the selected actinomycetes were tested by cross streak method. The two actinomycetes were streaked vertically and horizontally on

Kenknight's Agar medium. The plates were incubated for seven days, and observed for lysis at the juncture.

3.6.4. Compatibility between fungi and bacteria

The interaction between fungi and bacteria was studied adopting liquid culture co-inoculation method. Eight mm mycelial disc of one fungal endophyte was co-inoculated with a loopful of one bacterial endophyte in 100 ml of PDB and individual endophytes were inoculated separately for comparison. The bacteria and fungi were plated on their respective media after incubation periods of two and five days respectively. The microbial population in each plate was recorded and compared with that of individual cultures.

3.6.5. Compatibility between fungi, bacteria and actinomycetes

The fungi and bacteria selected from the above experiment were further tested for their compatibility with actinomycetes using liquid culture co-inoculation method. Eight mm discs of a selected fungus and an actinomycete along with one loopful of bacteria were inoculated simultaneously in 100 ml PDB and incubated at room temperature for seven days. Individual isolates inoculated in PDB were maintained separately. The bacteria, fungi and actinomycetes were plated on their respective media after two, five and seven days respectively to check for their compatibility and the compatible ones were selected for further studies.

3.7. CHARACTERIZATION AND IDENTIFICATION OF THE SELECTED ENDOPHYTES

The selected endophytes were identified based on cultural and morphological characters. Biochemical and molecular characterization were also carried out for bacterial isolates.

3.7.1. Identification of fungal endophytes

The fungal endophytes selected for microbial consortium were identified, based on cultural and morphological characters. The cultural characters like colour, growth, texture of the colonies and the morphological characters of mycelia, conidiophores, conidia etc. were recorded and compared with the original characters of the fungi and tentatively identified at genus level. For further confirmation and species level identification, the cultures were sent to National Centre for Fungal Taxonomy (NCFT), New Delhi.

3.7.2. Characterization of bacterial endophytes

The bacterial endophytes, VSB-1 and TRB-1, were characterized adopting the methods as suggested in the Manual of Microbiological Methods, published by the Society of American Bacteriologists (1957) and also by the Bergy's manual of Systematic Bacteriology, Vol I (Stanley *et al.*, 1989). The cultural, morphological, biochemical, and molecular characters of the isolates such as colony characters, pigment production, Gram's reaction, endospore staining, catalase reaction, indole production, gelatin liquefaction, starch and casein hydrolysis, denitrification, citrate utilization, lysine decarboxilase, ornithine decarboxilase, urease test, phenylalanine deamination, nitrate reduction, H_2S production and utilization of sugars *viz.* glucose, sucrose, fructose, lactose, arabinose, sorbitol and adonitol were studied. The Hi Assorted TM Biochemical test kit was also employed for the characterization and compared with the interpretation chart given in the manual. The molecular characterization was carried out by 16S rRNA sequencing to identify them to the species level. The BLASTn programme (http://ncbi.nlm.nhm.gov/blast) was used to find out the homology of the nucleotide sequence.

3.7.3. Identification of endophytic actinomycetes

The endophytic actinomycetes, ORA-1 and VRA-1 were identified based on cultural and morphological characters and the cultures were sent to NCFT, New Delhi for species level identification.

3.8. DEVELOPMENT OF MICROBIAL CONSORTIUM

Based on antagonistic potential, mutual compatibility, type and species of endophytic microorganisms, five microbial consortia were formulated consisting of four to six microorganisms (fungi, bacteria, and actinomycetes) in different combinations.

3.8.1. Evaluation of different consortia under *in planta* condition

Five different bioconsortia formulated were tested against *R. solanacearum* under *in planta* condition. Nursery was raised in earthern pots containing sterilized potting mixture using a highly susceptible variety, PKM-1 and 25 day old seedlings were transplanted in the polybags having sterilized potting mixture. Consortial inoculum was prepared by inoculating PDB with 48 h old bacterial culture (@ 1 loopful/100 ml) and five and seven day old fungal and actinomycete culture (@ 1 cm disc/100 ml) separately. The inoculation dates of different endophytes were adjusted accordingly to complete the incubation period of all endophytes on the same day. The cultures of the specific consortium were mixed and diluted with sterile water to prepare 30 per cent consortium suspension. This procedure was adopted for the preparation of consortial inoculum in all related experiments. These consortial suspensions were applied to the soil at the time of planting and challenge inoculation of the pathogen was done 30 days after planting (DAP). Each treatment was replicated thrice with 12 plants in each. The most effective consortium was selected based on the wilt incidence recorded at 7, 10 and 14 days after inoculation.

3.9. COMPARATIVE STUDY OF THE SELECTED MICROBIAL CONSORTIUM WITH INDIVIDUAL ANTAGONISTS ON BACTERIAL WILT INCIDENCE

A comparative study was conducted to evaluate the effect of selected microbial consortium along with individual endophytes on bacterial wilt incidence. The consortial inoculum was prepared as mentioned under 3.8.1. Application of microbial consortium and individual culture suspensions and challenge inoculation of the pathogen were done as mentioned earlier. Plants inoculated with the pathogen alone served as control. Observations were recorded on wilt incidence, plant height and days to flowering. Three replications were maintained with 12 plants in each replication.

3.9.1. Reisolation of endophytes

The endophytes were reisolated from soil, stem and root at 60 DAP and at the time of harvest on their respective media to confirm their endophytic nature and survivability in soil. The isolated colonies were compared with the original cultures to confirm their identity.

3.10. EVALUATION OF SELECTED ENDOPHYTIC CONSORTIUM UNDER POT CULTURE CONDITION

A pot culture experiment was carried out to study the efficacy of the selected microbial consortium adopting different methods of application using the highly susceptible variety, PKM-1. Earthern pots of size 9" x 9" were filled with solarised potting mixture consisted of soil, sand and cow dung @ 2:1:1. The chemical fertilizers were applied as per Package of Practices Recommendations of Crops (KAU, 2011).

The details of experiments are as follows:

Variety	: PKM-1
Design	: CRD
Number of replications	: 3
Number of treatments	: 8
Number of plants/treatment	: 12

Treatments

T_1- Seed treatment

T_2- Seedling dip

T_3- Soil drenching at the time of planting

T_4- Seed treatment + soil drenching at the time of planting

T_5- Seed treatment + seedling dip

T_6- Seed treatment + seedling dip+ soil drenching at 45 DAP

T_7- Control (with pathogen alone)

T_8- Absolute control

3.10.1. Preparation of microbial consortium inoculum

Microbial consortium of 30 per cent concentration was prepared as given under 3.8.1.

3.10.2. Methods of application

3.10.2.1. Seed treatment

One gram tomato seeds treated with 2 ml microbial consortial suspension for 30 min were air dried, and sown in earthern pots containing sterilized potting mixture.

3.10.2.2. Seedling dip

Twenty one day old seedlings were dipped in 30 per cent microbial consortium for 30 min before planting.

3.10.2.3. Soil drenching

Consortial suspension (30 %) was applied to soil @ 30ml/ plant at the time of planting in T_3 and T_4 treatments and at 45 days after planting in case of T_6.

3.10.3. Challenge inoculation

Challenge inoculation of the pathogen was done with fresh bacterial ooze suspension by soil drenching with wounding at 30 days after planting in all treatments except T_8 which served as absolute control without any treatment.

3.10.4. Observations

Observations on wilt incidence were recorded at 10, 14, and 21 days after inoculation. Biometric observations on plant height at 30, 45 and 60 days after planting, days to flowering and fruiting were also noted.

3.10.5. Reisolation of endophytes

The endophytes were reisolated from soil, stem and root from T_1, T_2 and T_3 treatments at 60 DAP and at the time of harvest on their respective media to confirm their endophytic nature and survivability in soil. The isolated colonies were compared with the original cultures to confirm their identity.

3.11. EFFECT OF MICROBIAL CONSORTIUM ON VIGOUR INDEX OF TOMATO SEEDLINGS

The selected endophytic consortium was assayed for growth promoting effect as suggested by Shende *et al.* (1977) and Elliot and Lynch (1984). One gram tomato

seeds were soaked in 2 ml of microbial consortium for 30 min and another set soaked in sterile water served as control. Air dried seeds were sown in pro-trays containing sterilised potting mixture. Observations on germination percentage, root and shoot length, and fresh and dry weight of the seedlings were recorded at 15 days after sowing.

Vigour index was calculated using the formula,

Vigour index = (Root length + shoot length) x germination percentage

3.12. STUDIES ON MECHANISM OF ANTAGONISM

To study the mode of action of the endophytes, production of volatile and non volatile metabolites, siderophore, indole acetic acid (IAA) and salicylic acid (SA) were estimated adopting standard protocols. In all experiments, 24 h, five and seven day old cultures of bacteria, fungi and actinomycetes respectively were used.

3.12.1. Production of volatile compounds by antagonistic endophytes

The endophytic isolates were tested for their ability to produce volatile metabolites to inhibit the growth of the pathogen by sealed Petri plate technique described by Dennis and Webster (1971a). Two Petri dish bases (9 cm) with one base poured with 15 ml of medium favouring the growth of the various antagonists (PDA for fungi, NA for bacteria, and KAM for actinomycetes) and other dish base was poured with NA to inoculate the pathogen, *R. solanacearum*. Eight mm disc of antagonistic fungus was placed at the centre of PDA mediated dish and in case of bacteria and actinomycetes, lawn were prepared on NA and KAM mediated dish respectively by spread plate method. The pathogen was point inoculated at the centre of NA mediated plate. The two dish bases were sealed together using adhesive tape with antagonist in the bottom plate. This allowed the pathogen growing in the upper plate to be exposed to volatile compounds produced by the antagonist. Similar sealed dishes with pathogen inoculated in upper dish and no antagonist in the lower, served

as control. Plates were incubated for 2-5 days depending on the antagonists. The colony diameter of the pathogen in treatments and control were measured and per cent inhibition of pathogen was calculated.

3.12.2. Production of ammonia by endophytic antagonists

The qualitative estimation of ammonia production was done following the method of Dye (1962) with slight modification. The selected endophytes were inoculated in 25 ml of peptone water (Appendix I) and incubated at 30^0C in shaker at 120 rpm for four days. After incubation, 0.5 ml of Nessler's reagent was added to the broth and noted for colour change in the broth from faint yellow to deep yellow or brown colour.

3.12.3. Production of hydrogen cyanide by endophytic organisms

Production of hydrogen cyanide (HCN) by the potential endophytes was detected following the method of Wei *et al.* (1991). The endophytic organisms were inoculated on King's B medium supplemented with 4.4 gL^{-1} of glycine. Sterile filter paper soaked in picric acid solution (picric acid 2.5 g + Na_2CO_3 12.5 g in 1000 ml water) was placed in the upper lid of Petri plates. Petri plates were sealed with adhesive tape to prevent volatilization and incubated for five days. Colour change of the filter paper from yellow to brown and to red indicate the production of HCN.

3.12.4. Production of non-volatile metabolites by endophytes

The effect of non-volatile metabolites of endophytic isolates on the growth of the pathogen was studied using culture filtrate and cellophane paper methods (Dennis and Webster, 1971b).

A. Culture filtrate method

The antagonistic endophytes were inoculated separately in 100 ml PDB and incubated at room temperature for 21 days. The mycelium and other cells were

removed by filtration through double layered filter paper and then sterilized by passing through bacterial proof filter of pore size 0.22 µm. Five per cent culture filtrate was added to 50 ml sterile medium and *R. solanacearum* was inoculated on the mediated plates by streaking. The media without filtrate served as control. Observation on the growth of the pathogen was recorded.

B. Cellophane paper method

In this method, cellophane paper (50 µm thickness) discs of 9 cm diameter were autoclaved at 1.1 kg cm^{-2} for 15 min and a sterilized disc was placed over PDA medium. Eight mm disc of each fungal endophyte was placed at the centre of the cellophane paper and incubated till full growth of the fungus was obtained. In case of bacteria and actinomycete, 0.1 ml suspension of the isolate was spread over the cellophane paper using a sterilized glass spreader and incubated for three and five days respectively. Later, the cellophane papers along with the adhering antagonists were removed carefully from the plates and a loopful of *R.solanacearum* was streaked on the media and compared with the growth in control plates.

3.12.5. Siderophore production

A. Iron dependant production of siderophores

King's B medium was amended with three different concentrations of $FeCl_3.6H_2O$ @ 0, 1 and 10 mg/l. The sterilized medium was poured into Petri plates and test cultures were streaked separately on the media and incubated for 48 h. Observations on production of greenish yellow fluorescent pigments were recorded (Kloepper *et al.*, 1980).

B. Screening on iron deficient media for siderophore

The bacterial cultures were grown on King's B medium and a loopful of test organisms were inoculated separately in 20 ml of iron deficient sodium succinate

broth (Appendix I) and incubated for 48 h at 28±2^{0}C with constant shaking at 120 rpm. Observation on presence of yellow green fluorescent pigment was recorded.

3.12.6. Production of IAA

The quantitative estimation of IAA produced by endophytic organisms was carried out adopting the colorimetric method of Gordon and Weber (1951).

In case of endophytic bacteria, a loopful of the culture was inoculated in 25 ml of Luria Bertani (LB) (Appendix I) broth supplemented with Tryptophan @ 100 µg ml^{-1} (100 µg ml^{-1} L-Tryptophan in 50% ethanol) as precursor and incubated for 24 h at room temperature on a rotary shaker. After incubation, the culture broth was centrifuged at 10,000 rpm for 15 min. To 2 ml of supernatant, two drops of ortho-phosphoric acid and 4 ml of Salkowsky reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35 % $HClO_4$) were added and incubated at room temperature for 30 min and the absorbance was read at 535 nm (Bric *et al.*, 1991).

IAA production by fungal endophytes was estimated as per the protocol suggested by Yadav *et al.* (2011). Three fungal discs of size 8 mm were inoculated in 100 ml of Czapek-Dox broth (Appendix I) amended with 1000µg ml^{-1} L-Tryptophan and incubated at room temperature for six days. Five ml of each culture was centrifuged at 10,000 rpm for 10 min. To 1 ml of supernatant, add 4-5 drops of ortho-phosphoric acid and 2 ml of Salkowsky reagent and mixed vigorously. The mixture was then incubated at room temperature for 30 min and absorbance measured at 540 nm using a spectrophotometer.

The quantitative estimation of IAA produced by endophytic actinomycete was determined according to the method suggested by Bano and Mussarat (2003). The actinomycete grown on Yeast extract-Malt Extract (YM) agar medium (Appendix I) and incubated at 30^{0}C for five days was added to 5 ml YM broth containing 2 mg ml^{-1} L-Tryptophan. The culture was incubated at 30^{0}C with shaking at 125 rpm

for seven days and then harvested by centrifugation at 11,000 rpm for 15 min. One ml of the supernatant was mixed with 2 ml of Salkowsky reagent and incubated at 28^0C for 30 min. The appearance of pink colour indicated IAA production. Optical density (OD) was read at 530 nm. A standard curve was drawn with different concentrations of IAA to determine the auxin production by the endophytic isolates.

3.12.7. Estimation of salicylic acid production in endophytic microorganisms

For quantitative estimation of salicylic acid (SA) production, the endophytic organisms were grown separately in 10 ml casamino acid broth (Appendix I) and incubated at 30^0C in the dark for 24 h, 72 h and seven days for bacteria, fungi and actinomycetes respectively at 2000 rpm in an orbital shaker (De Meyer and Hofte, 1997). Subsequently, 100µl of each culture was transferred separately to 25 ml of casamino acid broth and incubated as above. The cultures were centrifuged twice at 5000 rpm for 15 min and the supernatant was filtered through two layers of Whatman No.1 filter paper. The culture filtrate of each endophytic isolate was extracted with ethyl acetate and the organic phase was concentrated (1:3) under vacuum. SA concentration was determined by adding 5µl of 2M Fe Cl_3 and 3 ml of water to 1 ml of concentrated extract. The absorbance of the purple iron–SA complex, which developed in the aqueous phase was measured at 527 nm in a spectrophotometer and compared with a standard curve of SA dissolved in ethyl acetate.

3.13. EFFECT OF SECONDARY METABOLITES OF THE ENDOPHYTIC ORGANISMS ON *R. SOLANACEARUM*

The effect of secondary metabolites of the endophytes on *R. solanacearum* was evaluated under both *in vitro* and *in vivo* conditions.

3.13.1. Preparation of culture filtrate

The endophytic isolates were inoculated separately in 100 ml potato dextrose broth and incubated at 28 $\pm$ 2^0C for 21 days. The cell free culture filtrate of the

isolates was prepared by removing the mycelium and other cells by filtering first through double layered filter paper and then by passing through bacterial proof filter of pore size 0.22 µm.

3.13.2. *In vitro* evaluation of secondary metabolites of endophytes against *R. solanacearum*

For *in vitro* evaluation, *R. solanacearum* was inoculated on five per cent culture filtrate amended PDA medium. The medium without filtrate served as control. Observations on the growth of the pathogen were recorded.

3.13.3. Evaluation of secondary metabolites of the endophytes against *R. solanacearum* under *in planta* condition

In order to study the effect of secondary metabolites of endophytes on *R. solanacearum* under *in planta* condition, culture filtrates along with culture suspensions for comparison were used. Both undiluted and 30 per cent diluted culture filtrates were employed for the study.

3.13.4. Preparation of culture suspension

The endophytic bacteria, fungi, and actinomycetes were inoculated separately in 100 ml PDB and incubated for 2, 7 and 14 days respectively. These culture suspensions were diluted to the concentrations of 10^6 spores ml^{-1} for fungi, 10^8 cfu ml^{-1} for bacteria and 10^5 cfu ml^{-1} for actinomycete.

3.13.5. Preparation of inoculum

Wilted plants were collected, washed thoroughly in running tap water and the root portion was removed. 100 g of stem portion cut into smaller bits were suspended in 100 ml sterile water for 30 min and the concentration was adjusted to $OD_{600} = 0.3$.

3.13.6. Method of application

Healthy 30 day old seedlings were uprooted and the roots were washed thoroughly under tap water and then with sterile water. In the first experiment, the seedlings were given root dip in 30 per cent diluted culture filtrate of endophytic isolates for 2 h, then dipped in bacterial ooze suspension for 30 min and planted in polybags. Another set of seedlings were dipped in culture suspension of the endophytes for 2 h followed by dipping in ooze for 30 min before planting. In the second experiment, the seedlings were dipped first in ooze for 30 min and then in culture filtrate/suspension for 2 h before planting. Seedlings dipped in PDB and sterile water served as controls. The experiments were carried out in three replications with 12 plants in each treatment and wilt incidence was recorded at periodical intervals.

3.14. INDUCTION OF SYSTEMIC RESISTANCE IN TOMATO BY ENDOPHYTIC MICROBIAL CONSORTIUM

A pot culture experiment was conducted to study the changes in the defense mechanism in the root and stem of tomato plants due to application of endophytic microbes against *R. solanacearum*.

Variety	: PKM-1
Design	: CRD
Number of replications	: 3
Number of treatments	: 5
Number of plants/treatment	: 10

The treatments details are given below:

T_1: Microbial consortium alone

T_2: Microbial consortium +pathogen

T_3: Control (pathogen alone)

T_4: Medium alone

T_5: Absolute control

Consortial suspension 30 per cent was applied to the soil at the time of planting and challenge inoculation of the pathogen was done 30 DAP with fresh bacterial ooze. Plants in each treatment were carefully uprooted at 0, 5, and 10 days after inoculation of the pathogen, and the roots were washed in running tap water. These samples were stored at -80^{0}C for biochemical analysis.

3.14.1. Extraction and estimation of phenols in both root and stem of tomato

Phenols were extracted and analysed as described by Mahadevan and Sridhar (1982). Root and stem tissues of one gram each, were cut into small pieces and immersed in boiling absolute alcohol @ 10 ml alcohol/ 1 g of tissue and kept in boiling water bath for 5-10 min. After cooling, the tissues were crushed with sterilized mortar and pestle using 80 per cent alcohol and were filtered. Residues were again extracted with 80 per cent alcohol, boiling for 3 min and filtered. Both filtrates were mixed and final volume was made up to 4 ml with 80 per cent alcohol. Extracts were stored at 4^{0}C in screw capped vials covered with black paper. The whole extraction was done in dark to prevent light induced degradation of phenol.

A. Estimation of total phenols

Total phenol was estimated by Folin-Ciocalteau's method as described by Mahadevan and Ulaganathan (1991). To 1 ml of phenolic extract, 1 ml of Folin-Ciocalteau's reagent and 2 ml of 20 per cent Na_2CO_3 were added, shaken properly and heated on a boiling water bath for 1 min. Finally, the volume was adjusted to 25 ml with double distilled water. Absorbance was measured using spectrophotometer at 650 nm. Quantity of total phenol was estimated using catechol as standard.

B. Estimation of ortho-dihydroxy phenols (OD phenols)

OD phenol was estimated as described by Mahadevan and Ulaganathan (1991). To 1 ml of phenolic extract, 2 ml of 0.5 N HCl, 1 ml Arnow's reagent ($NaNO_3$-10g, Na_2MoO_4-10g, distilled water 100ml) and 2 ml of 1N NaOH were added. This was diluted to 10 ml with double distilled water. The tubes were shaken well and absorbance was recorded by spectrophotometer at 515 nm. Quantity of OD phenol was estimated using catechol as standard.

3.14.2. Assay of defense related oxidative enzymes

The important oxidative enzymes involved in the defense mechanism include phenylalanine ammonia-lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO). These enzymes were assayed at three intervals *viz.* 0, 5 and 10 days after inoculation of the pathogen.

A. Assay of peroxidase (PO)

One gram root and stem samples were homogenized separately in 2 ml 0.1 M phosphate buffer, pH 7.0 (Appendix II), at 4^0C. The homogenate was centrifuged at 16000 g at 4^0C for 15 min and the supernatant was used as the enzyme source. The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1 per cent H_2O_2. The changes in OD were recorded at 30 sec intervals for 3 min at 420 nm. The enzyme activity was expressed as changes in the OD min^{-1} g^{-1} protein (Hammerschmidt *et al.*, 1982).

B. Assay of polyphenol oxidase (PPO)

Polyphenol oxidase activity was determined as described by Mayer *et al.* (1965). Freeze dried root and stem samples of one gram each were homogenized in 2 ml 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16000 g for 15 min at

4^0C. The supernatant served as the enzyme source. The assay mixture comprised 0.2 ml of enzyme extract, 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 0.2 ml of 0.01 M catechol. The rate of increase in absorbance was recorded in 30 sec interval upto 3 min at 420 nm. The enzyme activity was expressed as changes in absorbance min^{-1} g^{-1} fresh weight of tissue.

C. Assay of phenylalanine ammonia-lyase (PAL)

One gram of root and stem samples were homogenized separately in 3 ml of ice-cold 0.1 M sodium borate buffer, pH 7.0 (Appendix II), containing 1.4 mM 2-mercapto ethanol and 0.1 g insoluble polyvinyl pyrrolidone. The extract was filtered through cheese cloth and the filtrate was centrifuged at 16000 g at 4^0C for 15 min. The supernatant was used as the enzyme source. Sample containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8, and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30^0C. OD value was recorded at 290 nm. Activity of phenylalanine ammonia-lyase was determined as trans-cinnamic acid as described by Dickerson *et al.* (1984). Enzyme activity was expressed as µmol trans-cinnamic acid min^{-1} g^{-1} protein.

3.14.3. Assay of pathogenesis related (PR) proteins

The major PR proteins involved in the defense mechanism are β-1,3-glucanase and chitinase. The changes in the activity of these enzymes were assayed in different intervals after the inoculation of the pathogen.

3.14.3.1. Assay of β-1,3-glucanase

β-1,3-glucanase activity was assayed by the laminarin dinitrosalicylic acid method (Pan *et al.*, 1991). One gram of tomato root and stem tissue were extracted separately in 5 ml of 0.05 M sodium acetate buffer (pH 5.0). The homogenates were centrifuged at 16000 g for 10 min at 4^0C and the supernatant was used as enzyme source. The enzyme extract (62.5 µl) was mixed with equal volume of 4 per cent

laminarin solution and incubated at 40^{0}C for 10 min. 375 µl of dinitrosalicylic acid was added to stop the reaction and heated for 5 min in boiling water bath. This coloured solution was diluted with 4.5 ml of distilled water, vortexed and its absorbance at 500 nm was determined. The enzyme activity was expressed as mg glucose released min^{-1} g^{-1} of sample.

3.14.3.2. Assay of chitinase activity in endophytic treated tomato plants

Chitinase enzyme activity was estimated according to the protocol described by Jeuniaux (1966) with slight modification.

Root and stem samples of one gram each were homogenized separately in 2 ml of 0.1 M sodium citrate buffer of pH 5.0 (Appendix II). The homogenates were centrifuged at 16000 g for 15 min at 4^{0}C and the supernatant was used for the enzyme assay.

A. Preparation of colloidal chitin

Two gram of pure chitin was dissolved in 64 ml of prechilled conc. H_2SO_4 with constant stirring and the temperature maintained at 4^{0}C. It was allowed to stand at 4^{0}C for 1 h with occasional stirring. This viscous solution was transferred to 90 ml prechilled distilled water with continuous stirring and kept overnight at 4^{0}C. The precipitate formed was centrifuged and resuspended in distilled water several times, to remove excess acid and then dialysed against tap water until pH reaches 5. Chitin content of the suspension was determined by drying the sample *in vacuo* and adjusted with distilled water to a final concentration of 10 mg ml^{-1} and stored at 4^{0}C for further use.

B. Preparation of P-dimethyl aminobenzaldehyde reagent (DAMB)

DMAB reagent was prepared by the procedure described by Reissig *et al.* (1955). Stock solution of DMAB was prepared by mixing 8 g of DMAB in 70 ml of

glacial acetic acid along with 10 ml of concentrated HCl. One volume of stock solution was mixed with 9 ml of glacial acetic acid immediately before use and the reagent was prepared fresh.

C. Colorimetric method of chitinase activity

The reaction mixture consisted of 10 µl of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme solution and 0.1 ml colloidal chitin. After incubation for 2 h at 37^0C, the reaction was stopped by centrifugation at 8000 g for 3 min. An aliquot of 0.3 ml supernatant was added into a glass reagent tube containing 30 µl of 1 M potassium phosphate buffer of pH 7.1 (Appendix II). After the addition of 2 ml of DMAB, the mixture was incubated for 20 min at 37^0C. Immediately thereafter, the absorbance was measured at 585 nm. N-acetyl glucosamine (G1cNAc) was used as a standard and the enzyme activity was expressed as moles G1cNAc equivalents $min^{-1}g^{-1}$ fresh weight.

3.15. FIELD EVALUATION OF MICROBIAL CONSORTIUM AGAINST BACTERIAL WILT DISEASE

A field experiment was conducted during October 2013 to January 2014 in wilt sick plot of Vellanikkara to evaluate the efficacy of the endophytic microbial consortium against bacterial wilt disease.

The details of the field experiment are as follows.

Varieties	: V_1 - PKM-1
	V_2 – COTH-3 (F1 hybrid)
	V_3 – Anagha
Design	: RBD
No. of replications	: 3
Plot size	: 8.64 m^2
Spacing	: 60 x 60 cm

No: of treatments : 15

No: of plants/treatment: 24

Treatment details:

T_1 – Endophytic microbial consortium (30 %)

T_2 – Rhizosphere microbial consortium (30 %)

T_3 –*Pseudomonas fluorescens* KAU reference culture (2 %)

T_4 – Soil drenching with copper hydroxide (2g/l)

T_5 – Control

Per cent wilt incidence was calculated using the formula

$$\text{Per cent wilt incidence} = \frac{\text{Number of plants wilted}}{\text{Total number of plants observed}} \times 100$$

3.15.1. Preparation of consortial suspension

Consortial suspension of endophytic isolates *viz. T. harzianum-1* (VSF-3), *T. viride-1* (CSF-1), *T. viride-2* (MyRF-1), *B. subtilis* (VSB-1), and *S. thermodiastaticus* (ORA-1) and consortial suspension of rhizosphere organisms *viz. T. harzianum* (CT-30) and *P. fluorescens* (VB-1) were prepared as mentioned in 3.8.1 and diluted to 30 per cent concentration.

3.15.2. Nursery preparation

The seeds of varieties *viz.* PKM-1, COTH-3 and Anagha were treated separately with 30 per cent endophytic and rhizospheric consortia and 2 per cent *P. fluorescens* for 30 min, air dried and sown in earthern pots containing sterilized potting mixture. The pots were kept in net house and irrigated regularly.

3.15.3. Methods of application

Based on the results of the pot culture experiment, seed treatment + seedling dip+ soil drenching at 45 DAP was adopted for the application of all biocontrol

treatments. Copper hydroxide @ 2 g/l was applied as soil drenching at the time of planting and 45 DAP. Soil drenching was given @ 6 l/m^2.

3.15.4. Field preparation

Experimental plots were prepared by ploughing followed by levelling and shallow trenches of length 2.7 m and width 4.5 cm were taken. Thirty day old seedlings were planted at a spacing of 60 cm between plants and 60 cm between rows. Agronomic practices were adopted as per Package of Practices Recommendations of Crops (KAU, 2011). Details of the treatments applied are mentioned in 3.15.

Another field experiment was carried out to study the effect of endophytic microbial consortium against bacterial wilt in moderately resistant variety (Mukthi) with two treatments consisted of endophytic consortium alone and a control.

3.15.4. Observations

Observations on wilt incidence at 30, 45 and 60 DAP, plant height, days to flowering, days to first harvest , number of fruits per plant, fruit yield per plant, average fruit weight and yield per plot were recorded.

3.15.5. Statistical analysis

Data were analyzed following analysis of variance for randomized block design (Gomez and Gomez, 1984). The post hoc test adopted was Duncan's Multiple Range Test (DMRT) to test the significance of experimental results.

Results

4. RESULTS

The experimental results obtained from the studies on "Enhancement of resistance to bacterial wilt in tomato by endophytic microbial communities" are presented below.

4.1. STANDARDIZATION OF DILUTION FACTORS FOR THE ISOLATION OF ENDOPHYTIC ORGANISMS

Preliminary isolation of endophytic bacteria, fungi and actinomycetes from root and stem samples of tomato plant were carried out using Vellanikkara samples, on their selective media, adopting standard protocols and the dilution factors for the isolation of endophytes were standardized. Dilutions of 10^{-4} and 10^{-3} which showed maximum countable colonies were selected for the isolation of endophytic bacteria from root and stem respectively, 10^{-2} and 10^{-1} dilution for endophytic fungi and actinomycetes respectively from both root and stem (Table 1).

4.2. ISOLATION AND ENUMERATION OF ENDOPHYTIC MICROBIAL POPULATION FROM COLLECTED SAMPLES

Endophytic microorganisms were isolated from both root and stem of healthy tomato plant samples collected from 16 locations (Plate 1A). Quantitative estimation of the endophytic microorganisms was carried out from these 32 samples, using the dilution factors standardized for each type of microorganism and data are presented in Table 2.

The isolated microbial population varied with the plant samples and the population was higher in root as compared to stem samples (Fig. 1). Microbial population varied significantly with the samples collected from different locations. Samples collected from various locations totally yielded bacterial, fungal and actinomycetes population of 1351×10^4 cfu g^{-1}, 272×10^2 cfu g^{-1}, 18×10^1 cfu g^{-1} and 832×10^3 cfu g^{-1}, 229×10^2 cfu g^{-1}, 3×10^1 cfu g^{-1} in root and stem respectively. Only

Table 1. Standardisation of dilution factor for the isolation of endophytic organisms

Dilutions	Endophytic population (cfu g^{-1} sample)					
	Bacteria		Fungi		Actinomycetes	
	Root	Stem	Root	Stem	Root	Stem
10^{-1}	Too numerous to count	Too numerous to count	29	22	3	1
10^{-2}	Too numerous to count	112	18	13	1	0
10^{-3}	180	44	2	0	0	0
10^{-4}	66	12	-	-	-	-
10^{-5}	19	3	-	-	-	-
10^{-6}	3	0	-	-	-	-

PLATE 1

A . COLLECTION OF SAMPLES

Vellanikkara

Mannuthy

Ozhalapathy

Eruthempathy

B. ISOLATION OF THE PATHOGEN

B . Bacterial wilt affected tomato plant

C. Colonies of *R. solanacearum*

Table 2. Endophytic microbial population of tomato collected from different locations

Sl. No	Location	Bacteria (cfu g^{-1})		Fungi (x 10^2 cfu g^{-1})		Actinomycetes (x 10^1 cfu g^{-1})	
		Root (x 10^4)	Stem (x 10^3)	Root	Stem	Root	Stem
1	Padannakkad (P)	68^{ef}	54^{bcd}	12^{d}	12^{de}	0	0
2	Panniyur (Py)	92^{d}	61^{abc}	26^{b}	13^{cde}	5	0
3	Malappuram (M)	52^{g}	44^{de}	22^{b}	12^{de}	0	0
4	Tavanur (T)	51^{g}	32^{fg}	14^{cd}	20^{ab}	0	0
5	Ozhalapathy (O)	82^{de}	68^{a}	14^{cd}	12^{de}	5	0
6	Eruthempathy(E)	73^{ef}	29^{g}	17^{c}	13^{cde}	0	0
7	Vellanikkara (V)	66^{ef}	44^{de}	18^{bc}	13^{cde}	3	1
8	Mannuthy (My)	63^{fg}	52^{cd}	13^{cd}	12^{de}	0	0
9	Cherumkuzhy (C)	71^{ef}	68^{a}	14^{cd}	15^{cde}	0	1
10	Ernakulam (Ek)	79^{de}	46^{de}	16^{cd}	23^{a}	0	0
11	Kumarakom (Ku)	138^{b}	64^{ab}	38^{a}	23^{a}	0	0
12	Alappuzha (A)	194^{a}	52^{cd}	14^{cd}	12^{de}	0	0
13	Kayamkulam (K)	118^{c}	64^{ab}	16^{cd}	11^{e}	0	0
14	Kallambalam (Ka)	80^{de}	46^{de}	14^{cd}	16^{bcd}	0	0
15	Vellayani (Vy)	52^{g}	68^{a}	12^{d}	11^{e}	0	0
16	Amburi (Am)	72^{ef}	40^{ef}	12^{d}	11^{e}	5	1
	Total	**1351**	**832**	**272**	**229**	**18**	**3**

Treatment means with same alphabet in superscript, do not differ significantly

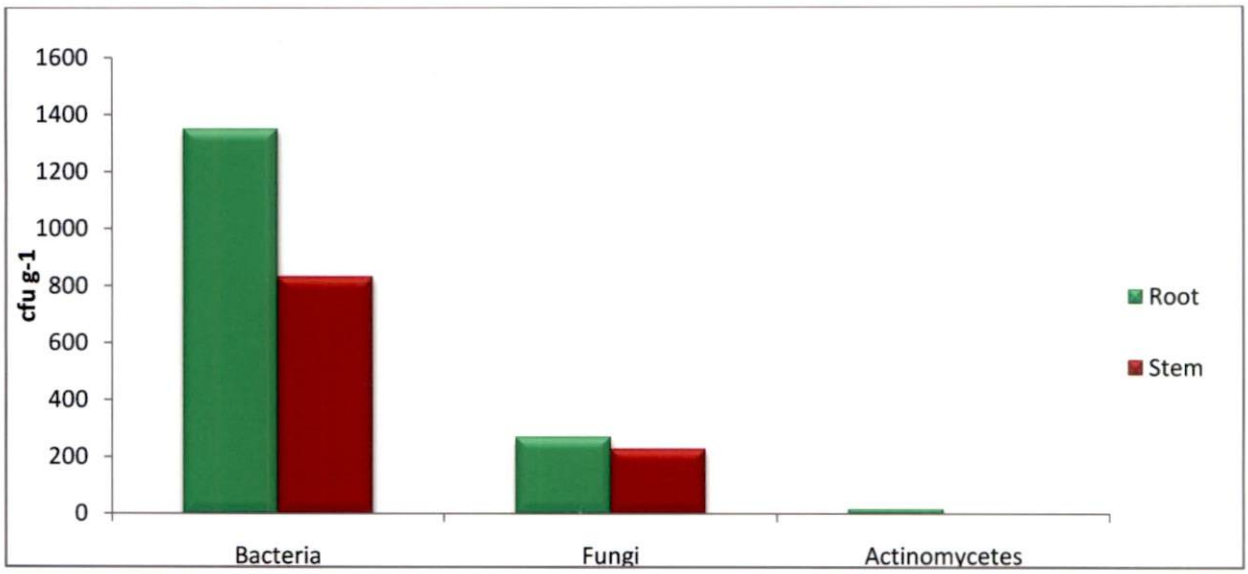


Fig. 1 Quantitative estimation of endophytic population in tomato root and stem samples

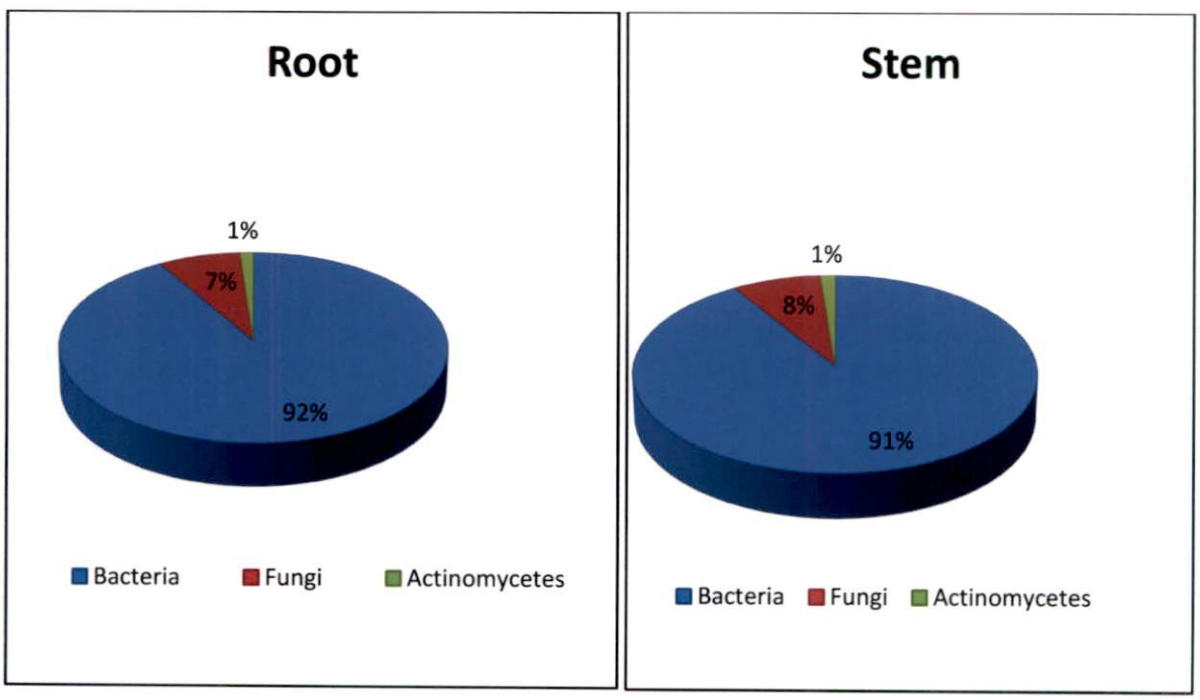


Fig. 2 Diversity of endophytic population in tomato root and stem samples

Vellanikkara and Amburi samples showed the presence of all three types of microorganisms in both root and stem.

Among the endophytic microorganisms isolated, bacterial population was higher than fungi and actinomycetes in both root and stem (Fig. 2). Bacterial population varied from 51 to 194 x 10^4 cfu g^{-1} in root and 29 to 68 x 10^3 cfu g^{-1} in stem samples among the different locations. Maximum population was recorded in the root sample collected from Alappuzha (194 x10^4 cfu g^{-1}) followed by Kumarakom (138 x 10^4 cfu g^{-1}) and in case of stem, samples collected from Cherumkuzhy, Ozhalapathy and Vellayani harboured maximum of 68 x 10^3 cfu g^{-1} (Fig. 3 &4).

Quantitative estimation of endophytic fungi revealed that, the population was comparatively less in root samples from all locations except in Kumarakom, Panniyur, and Malappuram which varied from 12 to 38 x 10^2 cfu g^{-1} root with maximum (38 x 10^2 cfu g^{-1}) in Kumarakom sample and in case of stem samples, it varied from 11 - 23 x 10^2 cfu g^{-1} with maximum in Ernakulam and Kumarakom (23 x 10^2 cfu g^{-1}) (Fig. 3 &4).

As compared to bacteria and fungi, actinomycete population was very less and only the root samples from Panniyur, Ozhalapathy, Amburi and Vellanikkara and the stem from Cherumkuzhy, Amburi and Vellanikkara showed actinomycete population. Among the different locations, the maximum population was present in root samples collected from Panniyur, Ozhalapathy and Amburi (5 x 10^1 cfu g^{-1}) (Fig. 3 &4).

From the enumerated microbial population, the predominant colonies of 154 microorganisms consisting of 79 bacteria, 68 fungi, and seven actinomycetes were selected. Among these, 44 bacteria, 42 fungi and four actinomycetes were from root and 35, 26 and three respectively from stem samples. Among the fungal endophytes, *Aspergillus* spp., *Penicillium* spp. and *Trichoderma* spp were the predominant ones. There were five fluorescent pseudomonads among the isolated endophytic bacteria.

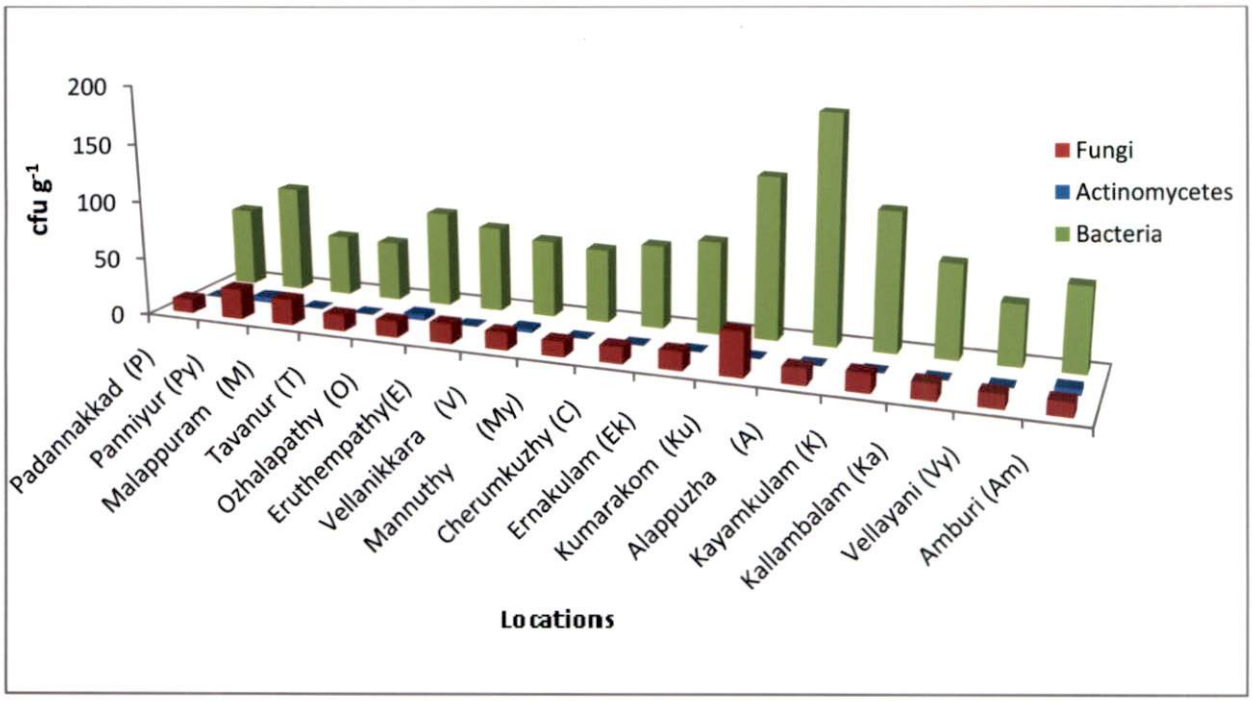


Fig. 3 Distribution of endophytes in root samples of tomato from different locations

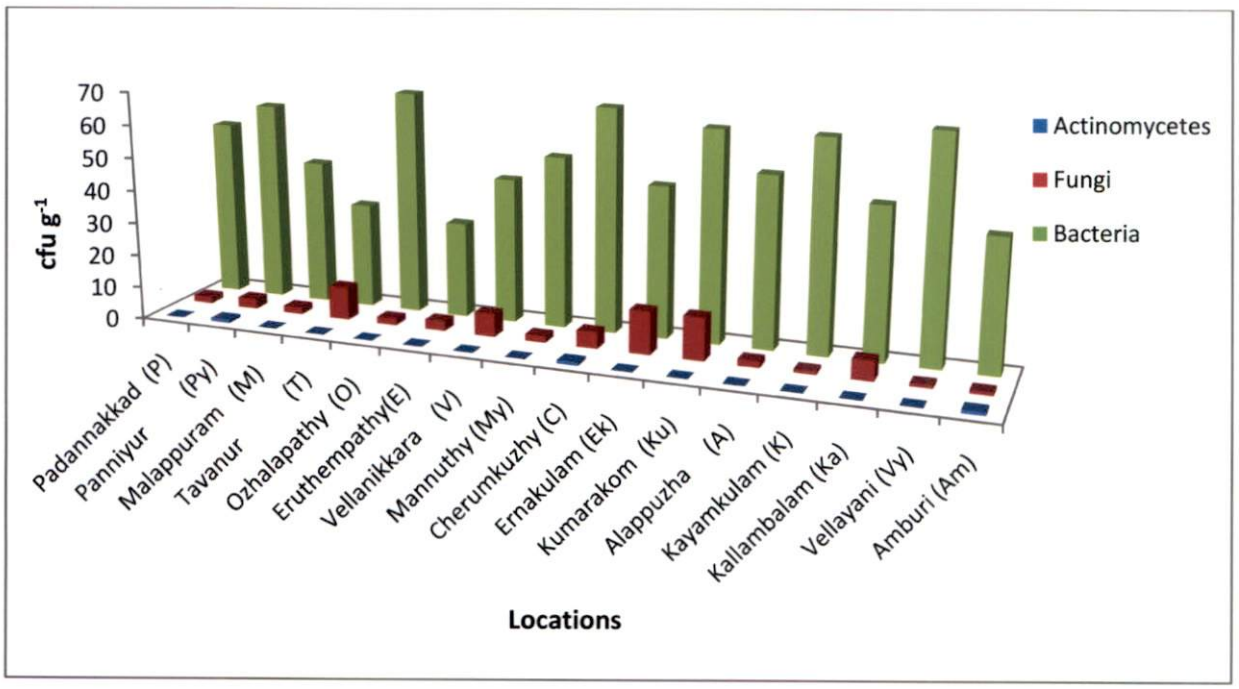


Fig. 4 Distribution of endophytes in stem samples of tomato from different locations

4.3. ISOLATION OF THE BACTERIAL WILT PATHOGEN

The pathogen isolated from wilted tomato plants on TZC medium yielded the typical colonies of *Ralstonia solanacearum* with circular, creamy white fluidal colonies with pinkish centre and the purified cultures were maintained for further studies (Plate 1B).

4.4. *IN VITRO* EVALUATION OF ENDOPHYTES AGAINST *R. SOLANACEARUM*

A preliminary *in vitro* screening was carried out with the isolated 154 endophytes including 79 bacteria, 68 fungi and seven actinomycetes (Plate 2A).

4.4.1. *In vitro* evaluation of bacterial endophytes against *R. solanacearum*

Among 79 bacteria screened, 31 isolates showed antagonistic reaction against *R. solanacearum*. It is observed from Table 3 that, per cent inhibition of the pathogen varied from 12.78 to 53.89 with maximum by EkRB-1 (53.89 %) followed by KuRB-3 (49.45 %) and MSB-1 (48.33 %) and the lowest inhibition was exhibited by AmRB-1 (12.78 %). Five isolates *viz.* OSB-3, KuRB-3, KuRB-4, KRB-2 and EkSB-1 also showed high pigment production. Based on per cent inhibition (> 40 %), 12 bacterial endophytes were selected for *in planta* studies.

4.4.2. *In vitro* evaluation of endophytic fungi against *R. solanacearum*

Endophytic fungi isolated from roots and stems of tomato plants were tested against *R. solanacearum* for their antagonistic property.

Out of 68 fungi tested, 27 showed antagonistic property against the pathogen and the results are presented in Table 4. It is found that, the per cent inhibition of the pathogen ranged from 11.67 to 66.67 with maximum by ARF-2 (66.67 %) followed by ERF-1 (60 %). The isolates PSF-1, MyRF-1, VyRF-1, ASF-3 and VSF-3 also showed antagonistic activity with more than 50 per cent inhibition. Most of the

Table 3. *In vitro* evaluation of bacterial endophytes against *Ralstonia solanacearum*

Sl. No	Isolates	* Per cent inhibition
1	PRB-1	40.56^{defg}
2	PyRB-2	13.63^{no}
3	PyRB-4	16.11^{lmno}
4	MRB-1	20.00^{kl}
5	MSB-1	48.33^{b}
6	TRB-1	41.11^{def}
7	TSB-1	14.78^{mno}
8	TSB-2	20.00^{kl}
9	TSB-3	18.33^{klmn}
10	ORB-1	35.00^{h}
11	ORB-2	36.11^{gh}
12	OSB-3	41.96^{de}
13	VSB-1	41.67^{de}
14	VSB-2	41.11^{def}
15	VRB-3	40.00^{efg}
16	VRB-4	18.89^{klm}
17	VRB-5	25.56^{ij}
18	MyRB-3	14.18^{mno}
19	CSB-1	43.07^{cde}
20	EkRB-1	53.89^{a}
21	EkSB-1	40.85^{def}
22	EkSB-2	33.07^{h}
23	KuSB-1	27.78^{i}
24	KuSB-2	36.67^{fgh}
25	KuRB-1	34.44^{h}
26	KuRB-3	49.45^{b}
27	KuRB-4	46.67^{bc}
28	KRB-2	45.00^{bcd}
29	KaSB-1	22.22^{jk}
30	AmRB-1	12.78^{o}
31	AmRB-3	14.74^{mno}

* Mean of three replications

Treatment means with same alphabet in superscript, do not differ significantly

Table 4. *In vitro* evaluation of fungal endophytes against *R. solanacearum*

Sl. No	Isolates	* Per cent inhibition	Mechanism	Other observations		
				Growth	Sporulation	Metabolite production
1	PRF-1	41.67^{fgh}	L	++	+	-
2	PRF-2	44.44^{fg}	L & O	+++	++	-
3	PSF-1	59.44^{abc}	O	++	++	++
4	PyRF-1	21.11^{jkl}	L & O	++	+	-
5	PyRF-2	36.11^{ghi}	L	++	+	-
6	MRF-2	16.67^{l}	L & O	+	++	++
7	TRF-2	44.44^{fg}	L & O	+++	++	+++
8	TRF-3	26.67^{ijk}	O	+++	++	-
9	TSF-1	21.11^{jkl}	L & O	+++	++	-
10	ESF-2	11.67^{l}	L	++	++	++
11	ERF-1	60.00^{ab}	L & O	++	++	-
12	VSF-I	27.22^{ijk}	L	+++	++	-
13	VSF-3	50.00^{cdef}	L & O	++++	+++	-
14	VRF-1	12.22^{l}	L	+++	+++	++++
15	MyRF-1	55.00^{bcd}	L	++	+	-
16	CRF-1	31.67^{i}	L & O	++	+++	-
17	CSF-1	42.78^{fg}	L & O	+++	+++	++++
18	EkSF-2	32.78^{hi}	L	+++	+++	-
19	ARF-1	30.00^{ij}	L & O	++	+	-
20	ARF-2	66.67^{a}	L & O	+++	+++	-
21	ASF-1	28.89^{ijk}	L	++	+++	-
22	ASF-2	45.56^{defg}	L & O	+++	+++	-
23	ASF-3	51.11^{bcdef}	L & O	++	++	-
24	VyRF-1	54.44^{bcde}	L	+++	+++	-
25	AmSF-1	19.44^{kl}	L	++	++	-
26	KaRF-3	45.00^{efg}	L & O	++	++	-
27	KaSF-2	43.33^{fg}	L	++	++	-

* Mean of three replications

Treatment means with same alphabets in superscript, do not differ significantly

Growth: ++++ - Very fast, +++ - Fast, ++ - Average, + - Slow

Sporulation: +++ - Good, ++ - Average, + - Poor

Metabolite: ++++ - Very high, +++ - High, ++ - Medium, - - Nil

L- lysis O-Overgrowth

isolates showed both lysis and overgrowth type of antagonism. Isolates PSF-1, MRF-2, TRF-2, ESF-2, VRF-1 and CSF-1 were found to produce metabolites. Based on per cent inhibition and metabolite production, 16 fungal endophytes were selected.

4.4.3. *In vitro* evaluation of endophytic actinomycetes against the bacterial wilt pathogen

Of the seven actinomycetes screened, only four isolates showed antagonistic activity against *R. solanacearum* of which the isolate, ORA-1 recorded maximum inhibition (27.22 %) and was on par with VRA-1 (26.67 %) (Table 5). All the four isolates, which showed inhibitory effect on the pathogen, were selected for *in planta* experiment.

4.5. SCREENING OF SELECTED ENDOPHYTES AGAINST THE BACTERIAL WILT DISEASE UNDER *IN PLANTA* CONDITION

Sixteen fungi, 12 bacteria and four actinomycetes selected from *in vitro* experiments were screened against the bacterial wilt pathogen under *in planta* condition using highly susceptible variety, PKM -1 and the wilt incidence recorded at 10 days after inoculation are furnished in Table 6, 7 and 8 (Plate 2B).

4.5.1. *In planta* screening of bacterial endophytes against bacterial wilt pathogen

Data presented in Table 6 showed that, all bacterial endophytes were superior to control in reducing wilt incidence. The incidence varied from 22.22 to 77.78 against 94.44 per cent in control at 10 DAI. The isolate, VSB-1 showed lowest incidence of 22.22 per cent followed by EkRB-1 and EkSB-1 (27.78 %) with 76.47 and 70.49 per cent efficiency over control respectively. Five bacterial isolates which showed less than 40 per cent incidence were selected for mutual compatibility test (Fig. 5).

Table 5. *In vitro* evaluation of endophytic actinomycetes against *R. solanacearum*

Sl. No	Isolates	Per cent inhibition
1	PyRA-1	21.11 [b]
2	ORA-1	27.22 [a]
3	VRA-1	26.67 [a]
4	AmRA-1	16.67 [b]

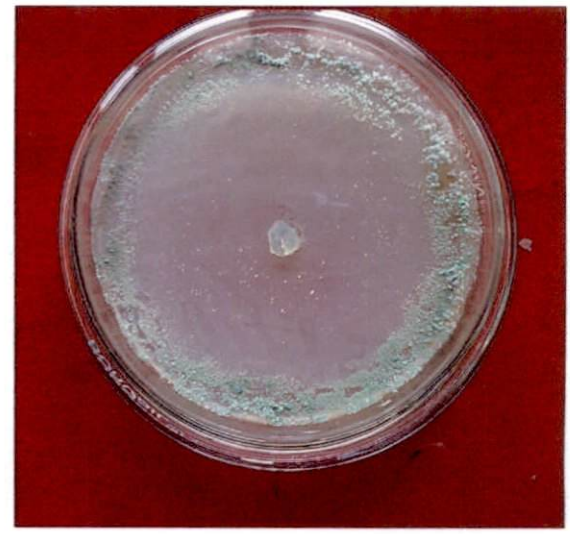
* Mean of three replications

Treatment means with same alphabet in superscript, do not differ significantly

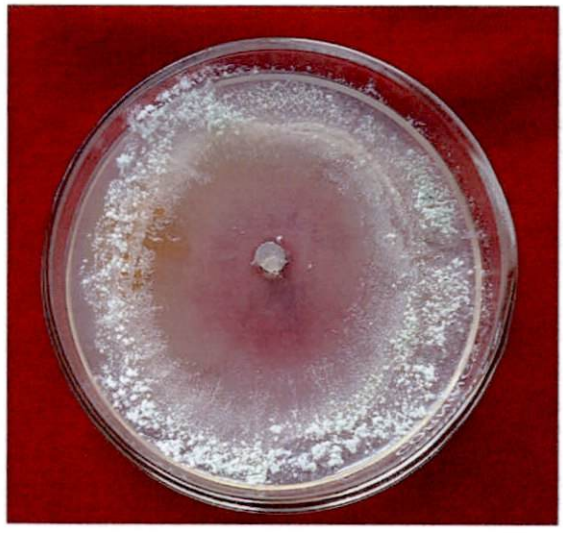
PLATE 2

A. *IN VITRO* EVALUATION OF ENDOPHYTES AGAINST *R.SOLANACEARUM*

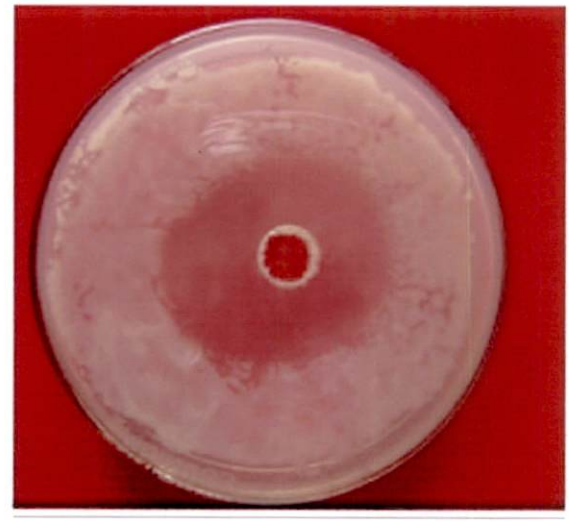
VSB-1 x *R.solanacearum*



VSF-3 x *R.solanacearum*



MyRF -1 x *R.solanacearum*



ORA -1 x *R.solanacearum*

B. *IN PLANTA* EVALUATION OF ENDOPHYTES AGAINST *R.SOLANACEARUM*

General view

Wilting symptom

Table 6. *In planta* screening of bacterial endophytes against *R. solanacearum*

Tr. No.	Bacterial endophytes	*Per cent wilt incidence 10 DAI	Per cent efficiency over control
1	PRB-1	44.44^{cde} (0.73)	52.94
2	MSB-1	44.44^{cde} (0.73)	52.94
3	TRB-1	38.89^{cde} (0.67)	58.82
4	OSB-3	77.78^{ab} (1.09)	17.64
5	VSB-1	22.22^{e} (0.48)	76.47
6	VSB-2	55.56^{bcd} (0.84)	41.17
7	CSB-1	38.89^{cde} (0.67)	58.82
8	EkRB-1	27.78^{de} (0.47)	70.59
9	EkSB-1	27.78^{de} (0.54)	70.59
10	KuRB-3	61.11^{bc} (0.90)	35.29
11	KuRB-4	66.67^{abc} (0.96)	29.41
12	KRB-2	50.00^{bcde} (0.79)	47.06
13	Control	94.44^{a} (1.29)	--

* Mean of three replications DAI – Days after inoculation

Treatment means with same alphabets in superscript, do not differ significantly
Figures in parenthesis are arc-sine transformed values

4.5.2. *In planta* evaluation of fungal endophytes against the pathogen

Data furnished in Table 7 revealed that, all fungal endophytes were superior to control in reducing disease incidence, which varied from 22.22 to 88.89 per cent against cent per cent in control at 10 DAI. Among the 16 fungal endophytes tested, all isolates except four showed high wilt incidence. MyRF-1 isolate showed minimum incidence of 22.22 per cent with 77.78 per cent efficiency over control and was on par with CSF-1, which showed 27.78 per cent incidence and 72.22 per cent disease reduction. The other two isolates, VSF-3 and ERF-1 showed 33.33 per cent incidence. Four isolates, which showed less than 40 per cent incidence, were also selected for further studies (Fig. 6).

4.5.3. *In planta* evaluation of endophytic actinomycetes against the pathogen

The four actinomycete isolates showed wilt incidence ranging from 33.33 to 88.89 per cent at 10 DAI with minimum by ORA-1 (33.33 %) recording 66.67 per cent efficiency over control and was on par with VRA-1 (38.89 %) showing 61.11 per cent disease reduction (Table 8). Isolates, AmRA-1 and PyRA-1 were least effective which recorded more than 80 per cent incidence (Fig. 7).

4.6. STUDIES ON MUTUAL COMPATIBILITY OF POTENTIAL ANTAGONISTS

The promising endophytes selected from *in planta* experiment were tested for their mutual compatibility to develop the microbial consortia. The interactions were studied among and between the three types of microorganisms *viz.* fungi, bacteria and actinomycetes (Plate 3).

4.6.1. Compatibility between fungal endophytes

Mutual compatibility between the selected eight fungal isolates was studied adopting dual culture technique and the observations recorded are presented in

Table 7. *In planta* evaluation of fungal endophytes against *R. solanacearum*

Tr. No	Fungal endophytes	*Per cent wilt incidence 10 DAI	Per cent efficiency over control
1	PRF-2	55.56^{cde} (0.84)	44.44
2	PSF-1	38.89^{de} (0.67)	61.11
3	MRF-2	66.67^{bc} (0.96)	33.33
4	TRF-2	88.89^{ab} (1.22)	11.11
5	ESF-2	61.11^{cd} (0.91)	38.89
6	ERF-1	33.33^{e} (0.61)	66.67
7	VSF-3	33.33^{e} (0.61)	66.67
8	VRF-1	38.89^{de} (0.67)	61.11
9	MyRF-1	22.22^{f} (0.48)	77.78
10	CSF-1	27.78^{f} (0.55)	72.22
11	ARF-2	72.22^{bc} (1.02)	27.78
12	ASF-2	61.11^{cd} (0.90)	38.89
13	ASF-3	38.89^{de} (0.67)	61.11
14	VyRF-1	77.78^{bc} (1.09)	22.22
15	KaRF-3	38.89^{de} (0.67)	61.11
16	KaSF-2	72.22^{bc} (1.02)	27.78
17	Control	100.00^{a} (1.37)	--

* Mean of three replications
DAI – Days after inoculation
Treatment means with same alphabets in superscript, do not differ significantly
Figures in parenthesis are arc-sine transformed values

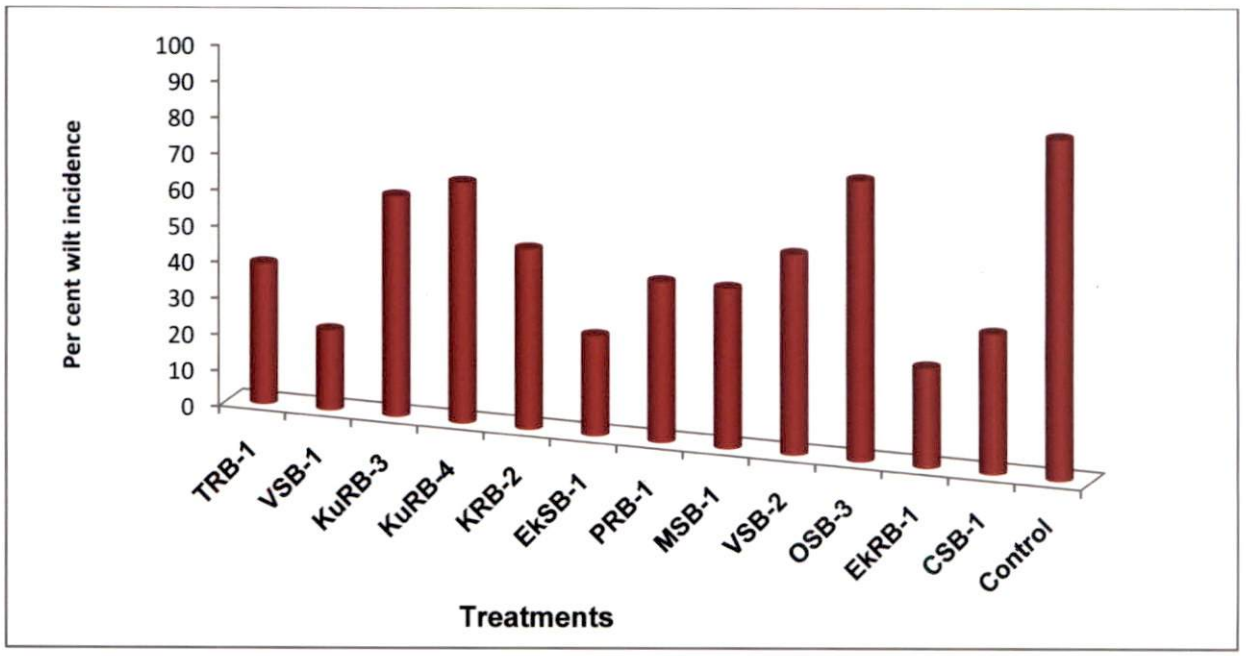


Fig. 5 *In planta* evaluation of bacterial endophytes against bacterial wilt pathogen

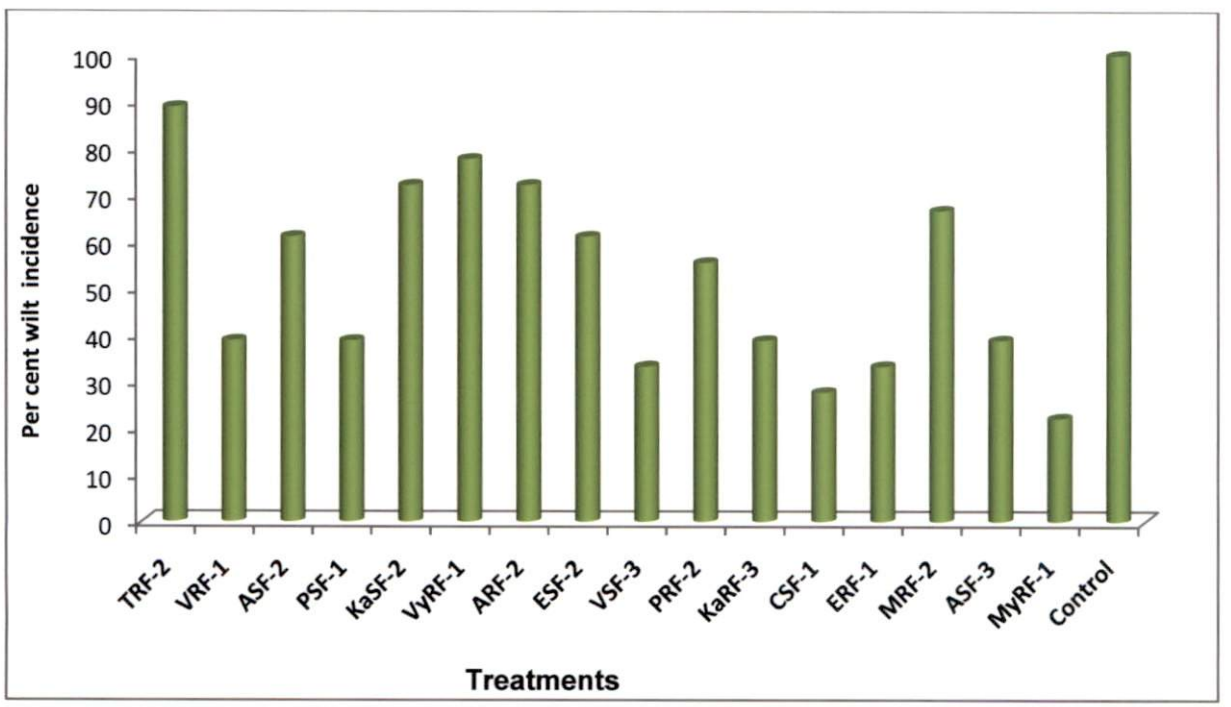


Fig. 6 *In planta* evaluation of fungal endophytes against bacterial wilt pathogen

Table 8. *In planta* evaluation of endophytic actinomycetes against *R. solanacearum*

Tr. No	Actinomycetes endophytes	*Per cent wilt incidence 10 DAI	Per cent efficiency over control
1	AmRA-1	83.33[a] (9.13)	16.67
2	ORA-1	33.33[b] (5.69)	66.67
3	VRA-1	38.89[b] (6.25)	61.11
4	PyRA-1	88.89[a] (9.45)	11.11
5	Control	100.00[a] (10.02)	--

* Mean of three replications DAI – Days after inoculation

Treatment means with same alphabets in superscript, do not differ significantly
Figures in parenthesis are square root transformed values

Table 9. There were total of 28 combinations among the fungi. The different interactions observed were intermingling of hyphae, presence of thick mycelial band, clear demarcation at the meeting point, heavy sporulation, and yellow pigmented band at the interaction site. Incompatible fungal combinations showed over growth, less sporulation and reduction in size of fungal colonies. Diffusion of metabolite was noticed in certain combinations. Other remarkable observations were the presence of thick brown pigmented band at the interaction point of CSF-1 and MyRF-1 on the reverse side of colonies, the colour change of spores of ASF-3 from green to yellow in ERF-1 x ASF-3 combination and the colour change from green spores of VSF-3 to olive green in MyRF-1 x VSF-3 combination. The highly compatible five isolates VRF-1, VSF-3, CSF-1, MyRF-1 and ASF-3 were selected for further studies.

4.6.2. Compatibility between bacterial antagonists

The five bacterial isolates selected from *in planta* experiment were subjected to mutual compatibility test by cross streak method. No lysis was observed at the juncture of TRB-1 x VSB-1, TRB-1 x EkRB-1 and VSB-1 x EkRB-1 combinations, which indicated the compatibility among the isolates and these three bacterial isolates EkRB-1, VSB-1 and TRB-1 were selected.

4.6.3. Compatibility between actinomycetes isolates

Two actinomycetes, ORA-1 and VRA-1 were found compatible, as no lysis was noticed at the juncture of the isolates.

4.6.4. Compatibility between fungi and bacteria

Growth characters of fungal and bacterial endophytes in liquid media were observed visually at second and seventh day of inoculation and the results are presented in Table 10.

Table 9. Mutual compatibility of endophytic fungal isolates

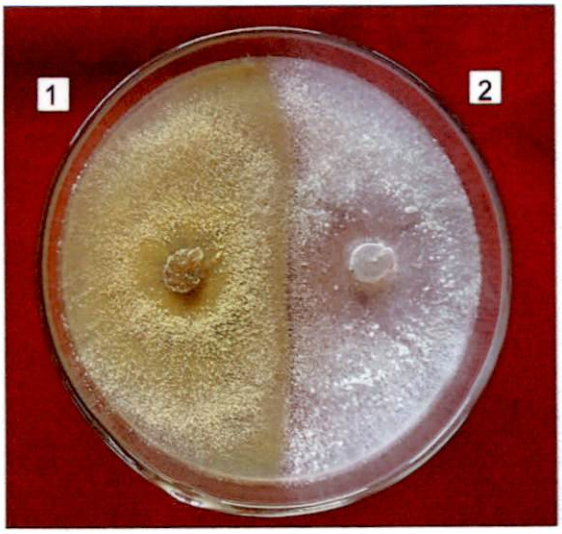
Sl. No.	Fungal combinations	Observations recorded
1	VRF-1 x PSF-1	Overgrowth, pigment diffuses to PSF-1
2	VRF-1 x VSF-3	Heavy sporulation at the meeting point
3	VRF-1 x KaRF-3	Overgrowth, pigment diffuses to KaRF-3
4	VRF-1 x CSF-1	Clear demarcation at the meeting point
5	VRF-1 x ERF-1	Clear demarcation at the meeting point, less sporulation in ERF-1
6	VRF-1 x ASF-3	Heavy sporulation at the interaction point
7	VRF-1 x MyRF-1	Heavy sporulation at the contact point, growth of MyRF-1 reduced
8	ERF-1 x PSF-1	Heavy sporulation at the interaction site, clear demarcation, heavy pigment production in PSF-1
9	ERF-1 x VSF-3	Intermingling of hyphae with heavy sporulation at the contact point, metabolite production in ERF-1
10	ERF-1 x KaRF-3	Overgrowth
11	ERF-1 x CSF-1	Overgrowth, growth of CSF-1 reduced
12	ERF-1 x ASF-3	Clear demarcation at the meeting point, pigment production in ASF-3, green coloured spores of ASF-3 turned yellow
13	ERF-1 x MyRF-1	Clear lytic zone at the meeting point, growth of MyRF-1 reduced
14	CSF-1 x PSF-1	Intermingling of hyphae with heavy sporulation at the contact point, metabolite production in CSF-1
15	CSF-1 x VSF-3	Clear demarcation at the meeting point
16	CSF-1 x KaRF-3	Overgrowth, growth of KaRF-3 reduced
17	CSF-1 x ASF-3	Clear demarcation at the meeting point, metabolite production in CSF-1
18	CSF-1 x MyRF-1	Thick brown pigmented band at the interaction point, less sporulation of CSF-1
19	PSF-1 x VSF-3	Clear lytic zone at the meeting point, yellow pigmented band present
20	PSF-1 x KaRF-3	Thick sporulation at the meeting point, growth of KaRF-3 reduced
21	PSF-1 x ASF-3	Yellow pigmented band at the interaction site, growth of ASF-3 reduced
22	PSF-1 x MyRF-1	Clear demarcation at the interaction point, growth of MyRF-1 reduced
23	KaRF-3 x VSF-3	Heavy sporulation at the meeting point, pigment production at the reverse side of VSF-3
24	KaRF-3 x ASF-3	Yellow pigmented band at the meeting point, growth of ASF-3 reduced
25	KaRF-3 x MyRF-1	Clear demarcation at the interaction point, growth of MyRF-1 reduced
26	ASF-3 x VSF-3	Clear demarcation at the meeting point, both cultures turned from dark to light green
27	ASF-3 x MyRF-1	Clear demarcation at the meeting point
28	MyRF-1 x VSF-3	Green spores of VSF-3 turned olive green, clear demarcation at the meeting point

Table 10. Mutual compatibility of endophytic fungal and bacterial isolates

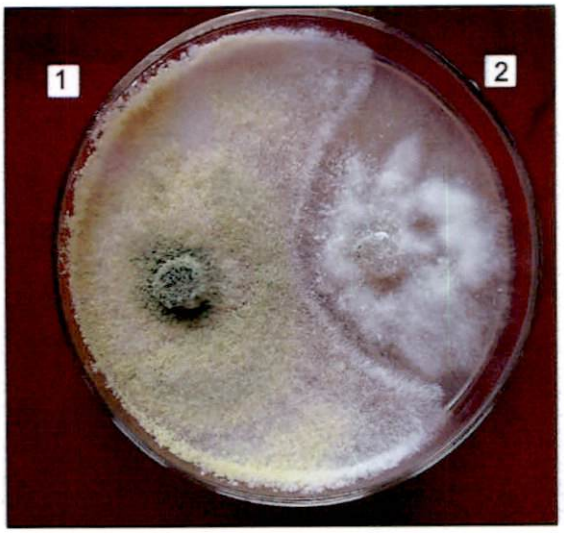
Sl. No.	Isolates	Mycelial growth	Sporulation	Bacterial growth	Filtrate colour
1	VSF-3 x VSB-1	+++	+++	+++	Colourless
2	VSF-3 x TRB-1	+	+	+++	Colourless
3	VSF-3 x EkRB-1	-	-	++	Colourless
4	VSF-3 (control)	+++	+++	-	Colourless
5	ASF-3 x VSB-1	+++	+++	+++	Colourless
6	ASF-3 x TRB-1	+	+	+	Colourless
7	ASF-3 x EkRB-1	-	-	+	Colourless
8	ASF-3 (control)	+++	+++	-	Colourless
9	CSF-1 x VSB-1	++	++	++	Brown
10	CSF-1 x TRB-1	++	++	+++	Light yellow
11	CSF-1 x EkRB-1	-	-	++	Brown
12	CSF-1 (control)	+++	+++	-	Brown
13	VRF-1 x VSB-1	++	+	++	Dark yellow
14	VRF-1 x TRB-1	+	+	+++	Light yellow
15	VRF-1 x EkRB-1	-	-	+	Light yellow
16	VRF-1 (control)	+++	+++	-	Dark yellow
17	MyRF-1 x VSB-1	+	+	++	Colourless
18	MyRF-1 x TRB-1	+	+	++	Colourless
19	MyRF-1 x EkRB-1	-	-	++	Colourless
20	MyRF-1 (control)	++	++	-	Colourless

Growth			Sporulation		
+++	-	Good	+++	-	Good
++	-	Average	++	-	Average
+	-	Poor	+	-	Poor
--	-	No growth	--	-	Nil

A. Fungus x Fungus

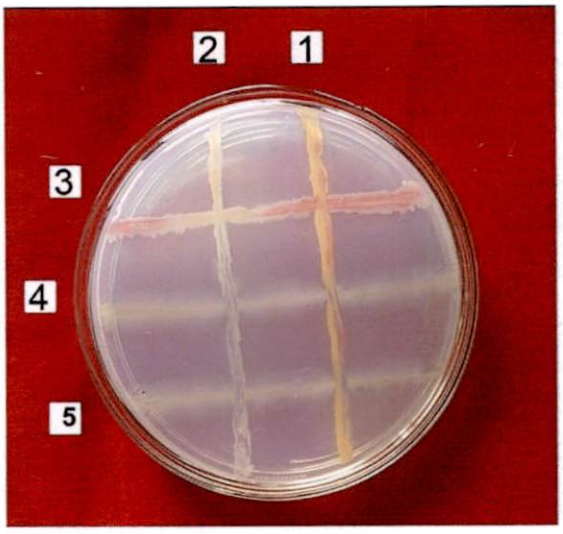


1.CSF-1 X 2. ERF-1



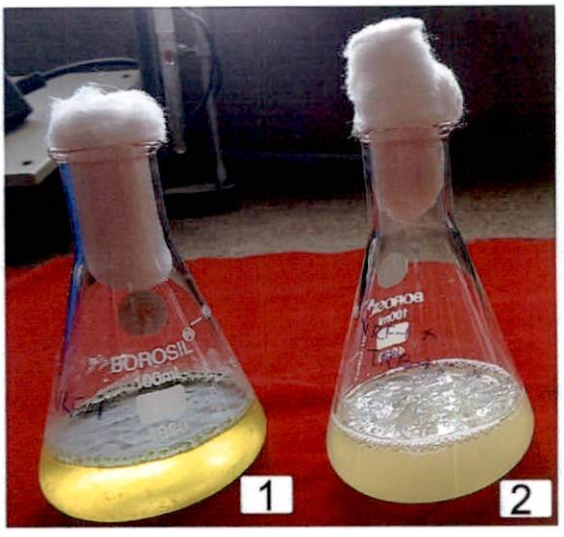
1. VSF-3 X 2. MyRF-1

B. Bacteria x Bacteria



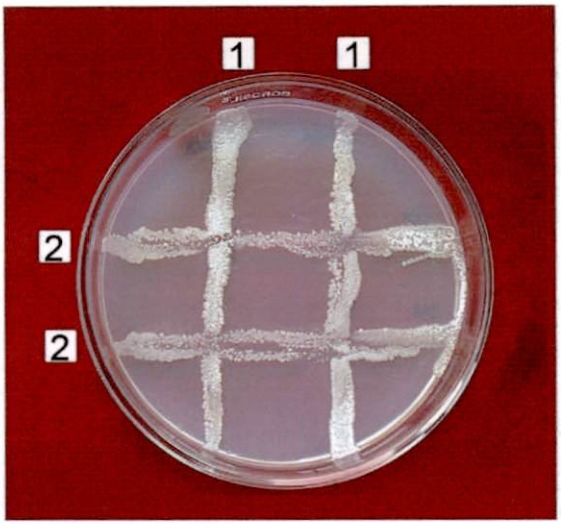
1. EkRB-1 2. VSB-1 3. TRB-1
4. MSB-1 5. CSB-1

C. Fungus x Bacteria



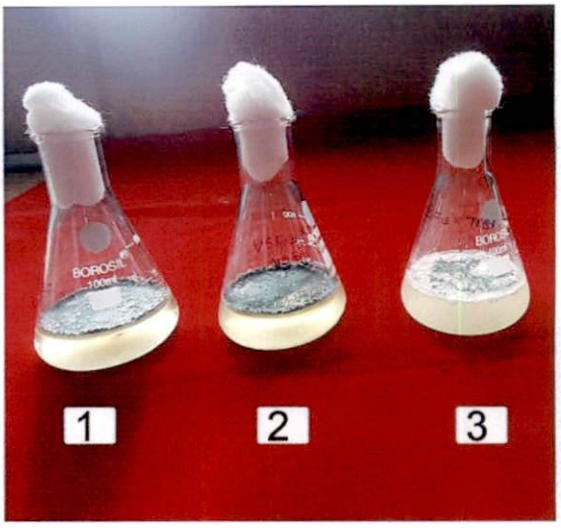
1.VRF-1 2.VRF-1 x TRB-1

D. Actinomycete x Actinomycete



1. ORA-1 2. VRA-1

E. Fungus x Bacteria x Actinomycete



1. VSF-3 2. VSF-3 x VSB-1 x ORA-1
3. VSF-3 x EkRB-1 x ORA-1

It is observed from the table that, the growth of fungal and bacterial antagonists varied with different combinations. Fungal growth and sporulation were affected by the combinations with bacterial isolate, EkRB-1, which indicated the incompatibility of this isolate with fungal endophytes. Plating of the cultures on their respective media also yielded high population of both fungi and bacteria except EkRB-1. All isolates except bacterial isolate EkRB-1 were selected for further studies.

4.6.5. Compatibility between fungi, bacteria and actinomycetes

Mutual compatibility between the selected fungi, bacteria, and actinomycetes was tested by co-inoculation in liquid media. Independent combinations with one each from the three organisms were inoculated in liquid medium. Sporulation and growth of the organisms were observed visually at two and seven days after inoculation for compatible combinations. It was further confirmed by plating on their respective media which also yielded high population of fungi, bacteria, and actinomycetes which indicated that the five fungal isolates *viz.* VRF-1, VSF-3, CSF-1, MyRF-1 and ASF-3, two bacteria, VSB-1 and TRB-1 and two actinomycetes, ORA-1 and VRA-1 were compatible to each other and therefore, all these isolates were selected for the development of different consortia.

4.7. CHARACTERIZATION AND IDENTIFICATION OF THE SELECTED ENDOPHYTES

Characters of the endophytes selected for the consortium were studied for the proper identification of the organisms.

4.7.1. Identification of the selected fungal endophytes

The selected fungal endophytes were identified based on their cultural and morphological characters (Plate 4A).

1. **Isolates, VSF-3 and ASF-3**

Cultural characters

Colony : Fast growth, the colour changes from white green to bright green with the sporulation in concentric rings.

Morphological characters

Mycelium : Hyaline, septate, smooth, 3.84-4.2 µm width.

Conidiophores : Loose tuft, main branch produced numerous side branches at right angles.

Phialides : Arise in false verticils upto five, short, skittle shaped, narrow at the base, bulged at the middle, attenuated abruptly into sharp pointed neck, 7.7-9.2 µm length.

Phialospores : Accumulated at the tip of phialides, sub-globose, short obovoid, often with truncate base, smooth, pale green, darker in mass, 1.35 µm in size.

Based on the above characters, these fungal isolates were tentatively identified as *Trichoderma harzianum* and confirmed from National Centre for Fungal Taxonomy (NCFT), New Delhi, ID No. NCFT- 4546.13 (VSF-3) and ID No. NCFT-4548.13 (ASF-3) (Plate 4a).

2. **Isolates, MyRF-1 and CSF-1**

Cultural characters

Colony : Fast growing, smooth surface, became hairy and colour changed from whitish green to dark green with maturity.

Morphological characters

Mycelium : Hyaline, smooth, septate, 2.86-3.2 µm width.

Conidiophores : Long and slender without sterile hyphae, side branches arise in compact tuft and all branches stand at wide angle.

Phialides : Not crowded and arise at right angles, attenuated into long neck, 8.3-9.4 µm length.

Phialospores : Globose, rough walled and green coloured, 2-3 µm in size.

Based on these characters, these fungal isolates were tentatively identified as *Trichoderma viride* and confirmed from NCFT, New Delhi, ID No. NCFT- 4545.13 (CSF-1) and ID No. NCFT- 4547.13 (MyRF-1) (Plate 4b & c).

3. **Isolate, VRF -1**

Cultural characters

Colony : Slow growth, initially white and become grayish green with centres raised and reverse yellow coloured. Colony size ranged from 2.9 to 3.8 cm.

Morphological characters

Mycelium : Septate, 5.8 – 6.4 µm width.

Conidiophore : 62 µm length.

Conidia : Globose, greyish green coloured, smooth and size 2.0-2.6 µm in size.

Based on these characters, the fungus was tentatively identified as *Penicillium melinii* and confirmed from Agarkhar Research Institute (ARI), Pune, ID No. ARI – 2.2 (Plate 4d).

4.7.2. Characterization of bacterial endophytes

Cultural, morphological and biochemical characters of the selected bacterial endophytes are presented in Table 11.

1. Isolate, VSB-1

Cultural characters : Colonies were large, irregular, dry, flat and cream coloured with undulate margin.

Morphological characters: Gram positive, rod shape, endospore formation.

Table 11. Cultural, morphological and biochemical characters of selected bacterial endophytes

Sl. No	Cultural/morphological/biochemical characters	VSB-1	TRB-1
1	Colony shape	Irregular	Round
2	Size	Large	Small
3	Margin	Undulate	Entire
4	Elevation	Flat	Umbonate
5	Colour	Cream	Red
6	Texture	Dry	Mucoid
7	Fluorescence	-	-
8	Pigment production	-	+
9	Grams staining	+	-
10	Shape	Rod	Rod
11	Endospore	+	-
12	Catalase	+	+
13	Indole production	-	-
14	Gelatin liquefaction	+	-
15	Starch hydrolysis	+	-
16	Casein hydrolysis	+	-
17	Denitrification	-	-
18	Citrate utilization	+	+
19	Lysine utilization	+	+
20	Ornithine utilization	+	+
21	Urease	-	-
22	Phenylalanine deamination	-	-
23	Nitrate reduction	+	+
24	H_2S production	-	-
	Utilization of sugars		
25	Glucose	+	+
26	Sucrose	+	+
27	Fructose	+	+
28	Lactose	+	-
29	Arabinose	+	+
30	Sorbitol	-	-
31	Adonitol	-	+

+ Positive reaction - Negative reaction

Table 12. Sequence analysis of endophytic bacteria

Isolate	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e-value
	Accession no:	Name				
VSB-1	CP006881.1	*Bacillus subtilis*	2423	99	98	0.0
	KF013234.1	*Bacillus* sp.	2423	99	98	0.0
	AB740156.1	*Bacillus subtilis*	2423	99	98	0.0
	KF053069.1	*Bacillus subtilis*	2423	99	98	0.0
	KC702829.1	*Bacillus* sp.	2423	99	98	0.0
TRB-1	KJ604964.1	*Serratia* sp.	318	100	99	9e-84
	KJ522787.1	*Serratia marcescens*	318	100	99	9e-84
	AB933259.1	*Pseudomonas fluorescens*	318	100	99	9e-84
	KJ722485.1	*Serratia ureilytica*	318	100	99	9e-84
	KJ607225.1	*Serratia rubidaea*	318	100	99	9e-84

Biochemical characters : Positive for biochemical tests including catalase, gelatin liquefaction, starch hydrolysis, casein hydrolysis, utilization of citrate, lysine and ornithine, nitrate reduction and utilization of glucose, sucrose, fructose, lactose and Arabinose. Negative for indole production, dentrification, urease and H_2S production. Based on these tests, the bacterium was tentatively identified as *Bacillus sp.*

Molecular characterization: The entire 16S rRNA gene sequence of the bacteria was analysed and the details are presented in Table 12. Based on sequence analysis, VSB-1 showed homology with *Bacillus subtilis* (Accession No. CP006881.1) having 99% query coverage and 98% identity (Plate 4e).

2. Isolate, TRB–1

Cultural characters : Colonies were round, small, mucoid red coloured with entire margin, pigment production.

Morphological characters : Gram negative, rod shape.

Biochemical characters : Positive for catalase, utilization of citrate, lysine and ornithine, nitrate reduction, utilization of glucose, sucrose, fructose, Adonitol and Arabinose. Negative for urease, H_2S production, starch hydrolysis, casein hydrolysis and gelatin liquefaction and indole production. Based on these tests, the bacterium was tentatively identified as *Serratia sp.*

Molecular characterization : The details of the analysis of entire 16S rRNA gene sequence of the bacteria, TRB -1 are presented in Table 12. Based on sequence analysis, TRB-1 showed homology with *Serratia marcesans* (Accession No. KJ522787.1) having 100% query coverage and 99% identity.

4.7.3. Characterisation of endophytic actinomycetes

The morphological and cultural characters of the actinomycetes were studied based on standard keys and the results are presented in Table 13.

The colony size of the isolates was medium. Both substrate and aerial mycelium were present. But chain of spores was absent in substrate mycelium of both

Table 13. Morphological and cultural characters of endophytic actinomycetes

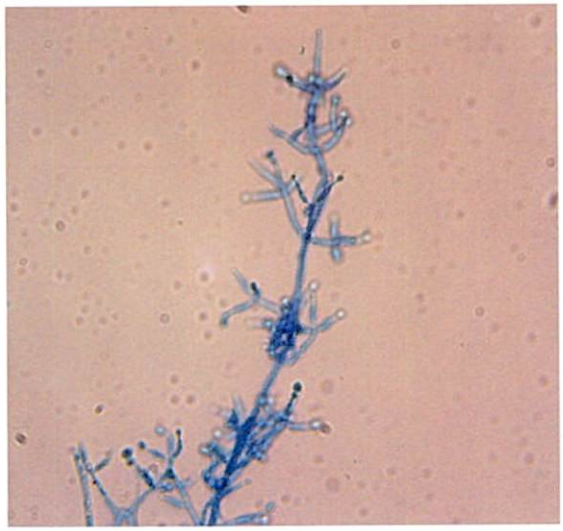
Sl No:	Characters	ORA-1	VRA-1
1	Colony size	Discrete	Discrete
2	Substrate mycelium	+	+
3	Chain of spores	-	-
4	Motile spores	-	-
5	Fragmentation of substrate mycelium	-	-
6	Aerial mycelium	+	+
7	Chain of arthrospores	+	+
8	Arthrospores in verticils	-	-
9	Spore chain morphology	Spirales	Spirales
10	Spore surface ornamentation	Smooth	Smooth
11	Color of spore mass	Gray	Gray
12	Pigmentation of substrate mycelium	-	-
13	Diffusible pigments	-	-
14	Probable genus	*Streptomyces*	*Streptomyces*

+ Presence - Absence

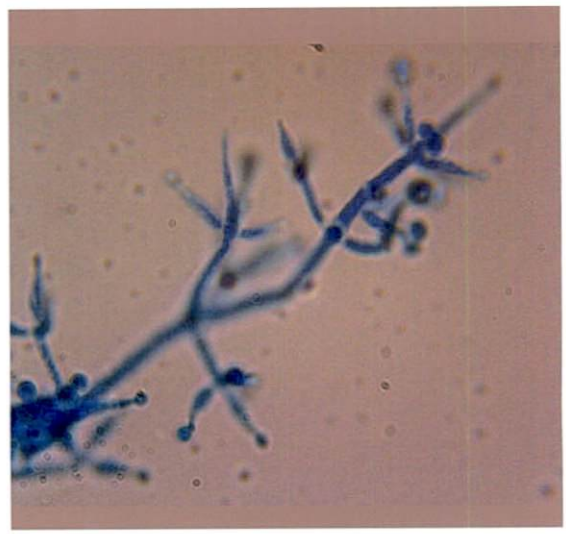
PLATE 4

IDENTIFICATION OF SELECTED ENDOPHYTES

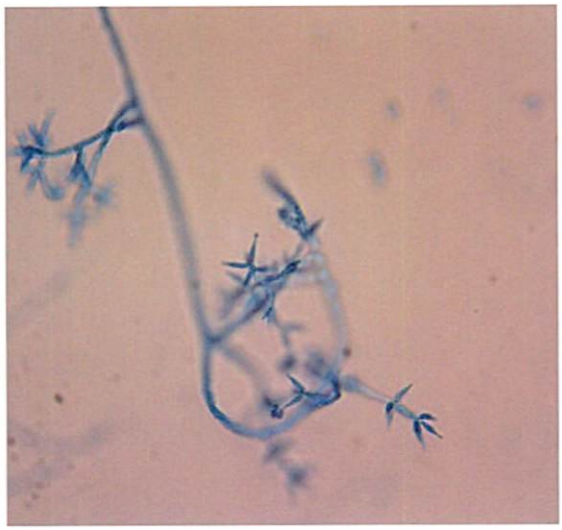
A. Fungi



a. Trichoderma harzianum (10 X 40x)



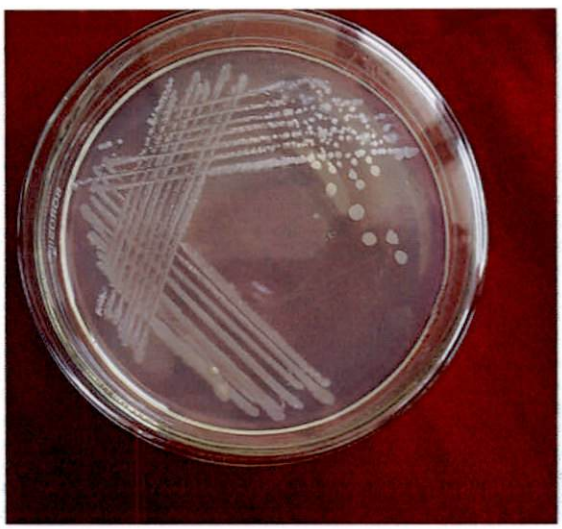
b. Trichoderma viride-1 (10 X 100x)



c. Trichoderma viride-2 (10 X 40x)

d. Penicillium melinii (10 X 100x)

B. Cultures of bacteria and actinomycete



e. Bacillus subtilis

f. Streptomyces thermodiastaticus

the isolates. Fragmentation of substrate mycelium and motile spores were absent for the isolates. Later the colonies of the isolates became covered with aerial mycelium bearing chain of arthrospores. Colour of the spore mass of both the isolates were gray. No pigment production was noticed for the isolates. Based on these cultural and morphological characters, the actinomycetes isolates were tentatively identified as *Streptomyces* spp. and confirmed ORA-1 as *Streptomyces thermodiastaticus* (ID No. 4287.11) (Plate 4f) and VRA-1 as *Streptomyces griseous* (ID No. 4285.11) by NCFT, New Delhi.

The identification details of the endophytes selected for microbial consortium are given in Table 14. It is also noted that, both root and stem portion of tomato plants harbour efficient antagonistic organisms, and also showed the presence of potential antagonists throughout Kerala, majority representing northern zone.

4.8. DEVELOPMENT OF MICROBIAL CONSORTIUM

Based on the ability to inhibit the pathogen under *in vitro* and *in planta* studies and mutual compatibility, nine endophytic isolates selected were identified for the development of various microbial consortia to evaluate their efficacy against the disease, of which TRB-1 was discarded due to ambilateral nature of the isolate (Table 14). Five different microbial consortia were developed with four to six isolates in different combinations and the details are given in Table 15.

4.8.1. Evaluation of different microbial consortia against bacterial wilt disease under *in planta* condition

Five different microbial consortia developed, were screened against the bacterial wilt disease under *in planta* condition. The observations on wilt incidence are furnished in Table 16. From the observations recorded at three intervals, it is noticed that, all treatments were superior to control. Among the five consortia, consortium No. 1 showed lowest wilt incidence of 10.32, 17.26 and 26.19 per cent at 7, 10 and 14 DAI respectively followed by consortium No. 5. It is also observed, at

Table 14. Identification of the endophytes selected for microbial consortium

Sl. No.	Isolates	ID-No.	Endophyte	Source
FUNGI				
1	CSF-1	NCFT- 4545.13	*Trichoderma viride*-1	Stem Cherumkuzhy
2	VSF-3	NCFT- 4546.13	*Trichoderma harzianum*-1	Stem Vellanikkara
3	MyRF-1	NCFT- 4547.13	*Trichoderma viride*-2	Root Mannuthy
4	ASF-3	NCFT- 4548.13	*Trichoderma harzianum*-2	Stem Alleppey
5	VRF-1	ARI – 2.2	*Penicillium melinii*	Root Vellanikkara
BACTERIA				
1	VSB-1	CP006881.1	*Bacillus subtilis*	Stem Vellanikkara
2	TRB-1	KJ522787.1	*Serratia marcesans*	Root Tavanur
ACTINOMYCETES				
1	ORA-1	NCFT- 4287.11	*Streptomyces thermodiastaticus*	Root Ozhalapathy
2.	VRA-1	NCFT- 4286.11	*Streptomyces griseous*	Root Vellanikkara

Table 15. Details on the combinations of different microbial consortia

Consortia No.	Endophytes
1	*T. harzianum*-1 (VSF-3) + *T. viride*-1 (CSF-1) + *T. viride*-2 (MyRF-1) + *B. subtilis* (VSB-1) + *S. thermodiastaticus* (ORA-1)
2	*T. harzianum*-1 (VSF-3) + *T. viride*-1 (CSF-1) + *T. viride*-2 (MyRF-1) + *B. subtilis* (VSB-1)
3	*T. harzianum*-1 (VSF-3) + *T. viride*-1 (CSF-1) + *T. viride*-2 (MyRF-1) + *Penicillium melinii* (VRF-1) + *B. subtilis* (VSB-1) + *S. thermodiastaticus* (ORA-1)
4	*T. harzianum*-1 (VSF-3) + *T. viride*-1 (CSF-1) + *T. viride*-2 (MyRF-1) + *T. harzianum* -2 (ASF-3) + *B. subtilis* (VSB-1)
5	*T. harzianum*-1 (VSF-3) + *T. viride*-1 (CSF-1) + *T. viride*-2 (MyRF-1) + *B. subtilis* (VSB-1) + *S. griseous* (VRA-1)

Table 16. *In planta* evaluation of microbial consortia against bacterial wilt of tomato

Consortia No.	Endophytes	Per cent wilt incidence		
		7 DAI	10 DAI	14 DAI
1	*T. harzianum*- 1 + *T. viride*- 1 + *T. viride*- 2 + *B. subtilis* + *S. thermodiastaticus*	10.32^{c} (0.34)	17.26^{c} (0.41)	26.19^{c} (0.53)
2	*T. harzianum*- 1 + *T. viride*- 1 + *T. viride*- 2 + *B. subtilis*	42.06^{b} (0.71)	47.62^{b} (0.76)	53.18^{b} (0.82)
3	*T. harzianum*- 1 + *T. viride* -1 + *T. viride*- 2 + *Penicillium melinii* + *B. subtilis* + *S. thermodiastaticus*	31.75^{bc} (0.60)	42.06^{b} (0.71)	57.94^{b} (0.87)
4	*T. harzianum*- 1 + *T. viride* -1 + *T. viride*- 2 + *T. harzianum* -2 + *B. subtilis*	42.86^{b} (0.71)	42.86^{b} (0.71)	53.97^{b} (0.83)
5	*T. harzianum*- 1 + *T. viride*- 1 + *T. viride*- 2 + *B. subtilis* + *S. griseous*	26.19^{bc} (0.53)	31.75^{bc} (0.60)	47.62^{b} (0.76)
	Control (without treatment)	83.33^{a} (1.16)	83.33^{a} (1.16)	100.00^{a} (1.37)

DAI- Days after inoculation

Treatment means with same alphabets in superscript, do not differ significantly
Figures in parenthesis are arc-sine transformed values

14 days after inoculation, consortium No.1 showed effective management of disease recording only 26.19 per cent against cent per cent in control (Fig. 8). Based on these results, consortium No.1 consisted of three fungi *viz. T. harzianum*-1 (VSF-3), *T. viride*- 1 (CSF-1) and *T. viride*- 2 (MyRF-1), one bacteria *viz. B. subtilis* (VSB-1) and one actinomycete, *Streptomyces thermodiastaticus* (ORA-1) was selected for further studies.

4.9. COMPARATIVE STUDY OF SELECTED MICROBIAL CONSORTIUM WITH INDIVIDUAL ANTAGONISTIC ENDOPHYTES ON BACTERIAL WILT INCIDENCE AND BIOMETRIC CHARACTERS OF TOMATO

On evaluation of selected consortium with individual endophytes, it is noted from Table 17 that, combined application of endophytes was significantly superior to individual application, as it showed minimum incidence of 12.5 per cent with 73.21 per cent reduction over control. Among the individual applications, incidence varied from 22.22 to 44.44 per cent with minimum in *T. viride*-2 (22.22%) followed by *B. subtilis* and *T. viride*-1 with 27.78 per cent and were superior to control.

With respect to biometric characters, significant difference was noticed in plant height with maximum recorded in treatment with consortium recording 87.79 cm against 69.69 cm in control at 60 days after planting. The application of consortium also showed early flowering (35.77 days) as compared to individual applications and control. Not much variation was noticed between individual treatments and the control with respect to days to flowering.

4.9.1. Reisolation of endophytes

Reisolation of individual endophytes yielded high population of each organism from soil, root, and stem at 60 DAP. Soil samples showed *Trichoderma* spp, *Bacillus* and *Streptomyces* population of 6-14 x 10^4, 76 x 10^8 and 15 x 10^5 cfu g^{-1} whereas reisolation from root and stem yielded 3-6 x 10^1, 39 x 10^4 and 8 x 10^1 cfu g^{-1}

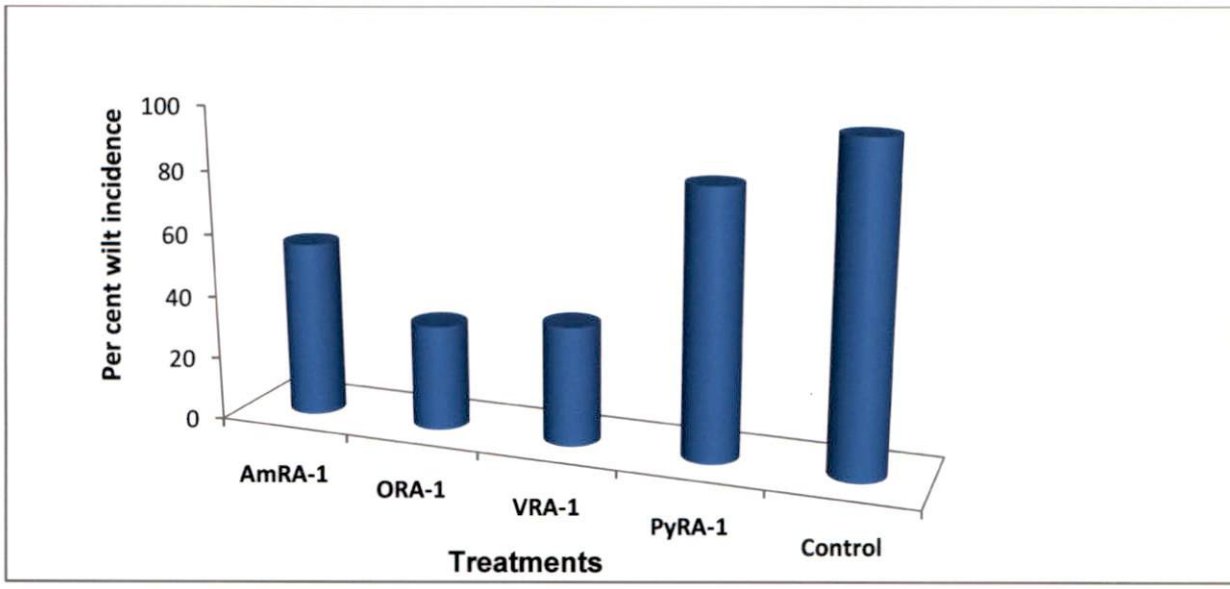


Fig. 7 *In planta* evaluation of actinomycete endophytes against bacterial wilt pathogen

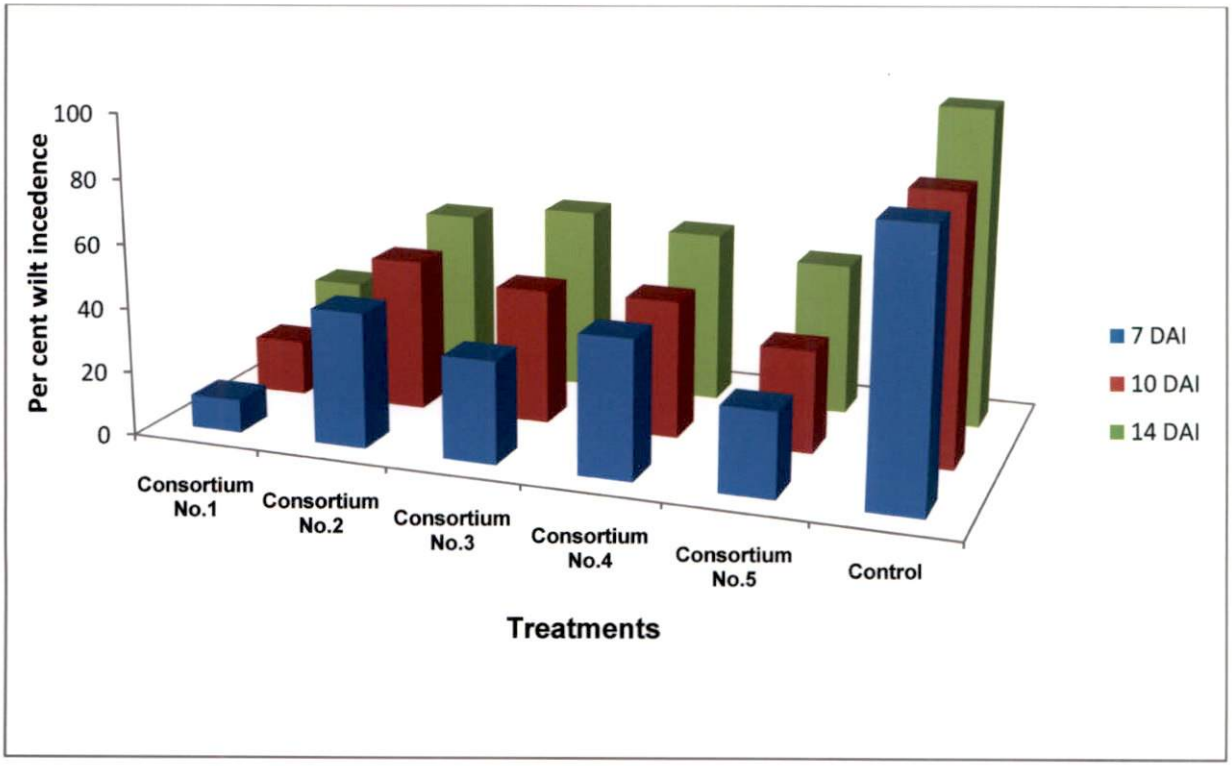


Fig. 8 *In planta* evaluation of bioconsortia against bacterial wilt pathogen

Table 17. Comparison of endophytic consortium with individual isolates on bacterial wilt and biometric characters of tomato

Tr. No.	Isolates	Per cent wilt incidence 10 DAI	Per cent reduction over control	Plant height (cm)			Days to flowering
				Days after planting			
				30	45	60	
1	Endophytic consortium	12.5^{d} (0.35)	73.21	44.76^{a}	69.73^{a}	87.79^{a}	35.77
2	*B. subtilis*	27.78^{bcd} (0.55)	55.75	37.71^{b}	62.43^{b}	80.43^{b}	37.72
3	*S.thermodiastaticus*	44.44^{b} (0.73)	36.71	29.05^{ef}	53.76^{de}	71.74^{de}	41.12
4	*T. viride* -1	27.78^{bcd} (0.55)	55.75	32.71^{cd}	57.19^{cd}	75.36^{cd}	40.12
5	*T. harzianum*-1	33.33bc (0.61)	49.40	35.33^{bc}	59.83^{bc}	77.97^{bc}	37.59
6	*T. viride*-2	22.22^{cd} (0.49)	62.10	31.29^{de}	56.07^{cd}	74.27^{cd}	39.21
7	Control	87.5^{a} (1.22)	--	26.52^{f}	51.52^{e}	69.69^{e}	41.92

DAI – Days after inoculation

Treatment means with same alphabets in superscript, do not differ significantly
Figures in parenthesis are arc-sine transformed values

Table 18. Reisolation of endophytes from individual application

Endophytes	60 Days after planting			At the time of harvest		
	Soil (cfu g^{-1})	Root (cfu g^{-1})	Stem (cfu g^{-1})	Soil (cfu g^{-1})	Root (cfu g^{-1})	Stem (cfu g^{-1})
T. harzianum-1	14×10^4	6×10^1	3×10^1	11×10^4	5×10^1	1×10^1
T. viride-1	6×10^4	4×10^1	4×10^1	3×10^4	2×10^1	3×10^1
T. viride-2	12×10^4	3×10^1	1×10^1	5×10^4	3×10^1	1×10^1
B. subtilis	76×10^8	39×10^4	22×10^3	48×10^8	30×10^4	20×10^3
S. thermodiastaticus	15×10^5	8×10^1	5×10^1	10×10^5	6×10^1	3×10^1

and 1-4 x 10^1, 22 x 10^3 and 5 x 10^1 cfu g^{-1} respectively. At the time of harvest, the population of *Trichoderma* spp, *Bacillus* and *Streptomyces* were found to be 3-11 x 10^4, 48 x 10^8 and 10 x 10^5 cfu g^{-1} in soil, 2-5 x 10^1, 30 x 10^4 and 6 x 10^1 cfu g^{-1} in root and 1-3 x 10^1, 20 x 10^3 and 3 x 10^1 cfu g^{-1} in stem respectively and showed much reduction in bacterial population (Table 18). Comparison of the reisolated organisms with their original cultures and microscopic observation showed similarity and thus confirmed their endophytic nature and survivability in the soil.

4.10. EVALUATION OF THE SELECTED MICROBIAL CONSORTIUM AGAINST BACTERIAL WILT DISEASE UNDER POT CULTURE CONDITION

A pot culture experiment was carried out to evaluate the efficacy of the selected microbial consortium by adopting different methods of application. Observations on per cent wilt incidence and biometric characters were recorded (Plate 5).

4.10.1. Effect of consortial treatment on bacterial wilt incidence

Observations on wilt incidence recorded at 10, 14 and 21 days after inoculation is summarized in Table 19. The data indicated that, all treatments were superior to control at all the three intervals. At 10^{th} day after inoculation, incidence was very less, ranged from 0 to 8.33 per cent in different treatments against 27.78 in control (T_7). Among the treatments, T_6 (seed treatment + seeding dip + soil drenching 45 DAP) recorded lowest incidence at 14 (5.55 %) and 21 (11.11 %) days after inoculation against 61.11 and 80.55 per cent in control (Fig. 9). All other treatments belonged to a homogenous subgroup. Based on these results, seed treatment + seeding dip + soil drenching 45 DAP was selected as the method of application in the field experiments.

PLATE 5

POT CULTURE EXPERIMENT

T_1 - Seed treatment T_2 -Seedling dip

T_3 - Soil application at the time of planting

T_5 - Seed treatment + Seedling dip

T_6 - Seed treatment + Seedling dip + 45 DAP

T_7- Control (with pathogen)

T_8-Absolute control

Table 19. Evaluation of selected microbial consortium against bacterial wilt of tomato in pot culture

Sl No.	Treatments	Per cent wilt incidence		
		10 DAI	14 DAI	21 DAI
1	T_1 - Seed treatment	5.56	16.67^{b} (4.05)	22.22^{bc} (4.61)
2	T_2 - Seedling dip	0	8.33^{ab} (2.61)	16.66^{ab} (3.92)
3	T_3 - Soil application at the time of planting	8.33	16.67^{b} (4.05)	22.22^{bc} (4.61)
4	T_4 - Seed treatment + Soil application at the time of planting	0	13.89^{ab} (3.17)	19.44^{b} (4.31)
5	T_5 - Seed treatment + Seedling dip	0	8.33^{ab} (2.61)	13.89^{ab} (3.17)
6	T_6 - Seed treatment + Seedling dip + Soil application at 45 DAP	0	5.55^{ab} (2.22)	11.11^{ab} (2.99)
7	T_7 - Control (with pathogen)	27.78	61.11^{c} (7.84)	80.55^{c} (9.00)
8	T_8 - Absolute control	0	0^{a} (0.71)	0^{a} (0.71)

DAI – Days after inoculation
Treatment means with same alphabets in superscript, do not differ significantly
Figures in parenthesis are square root transformed values

4.10.2. Effect of consortial treatment on biometric characters of tomato

Biometric characters including plant height, days to flowering and fruiting were recorded for each treatment (Table 20). With respect to plant height, much variation was not observed with different treatments at three intervals. The maximum plant height of 82.59 cm was noticed in T_6 against 72.5 cm in control at 60 days after planting. Days to flowering and fruiting ranged from 37.57 to 41.87 days and 46.89 to 51.19 days respectively and were found to be non-significant. T_6 showed early flowering (37.57 days) and fruiting (46.89 days) as compared to other treatments.

4.10.3. Reisolation of endophytes

The endophytes were reisolated from soil, root, and stem at 60 DAP and harvest on their respective selective media and the result is shown in Table 21. Reisolation from soil samples showed higher population of endophytic *Trichoderma* spp, *Bacillus* and *Streptomyces* recording 22 x 10^4, 68 x 10^6 and 13 x 10^5 cfu g^{-1} respectively and the endophytic population reisolated from root and stem were comparatively less showing 14 x 10^1, 44 x 10^4, and 6 x 10^1 cfu g^{-1} and 6 x 10^1, 28 x 10^3 and 3 x 10^1 cfu g^{-1} respectively. At the time of harvest, the population of *Trichoderma* spp, bacteria and actinomycetes were found to be 19 x 10^4, 48 x 10^8 and 10 x 10^5 cfu g^{-1} in soil, 10 x 10^1, 30 x 10^4 and 5 x 10^1 cfu g^{-1} in root and 5 x 10^1, 20 x 10^3 and 3 x 10^1 cfu g^{-1} in stem respectively (Table 21). Comparison of the reisolated organisms with the original cultures of the same and microscopic examination confirmed the endophytic nature of the organisms.

4.11. DETERMINATION OF VIGOUR INDEX

Vigour index of tomato seedlings treated with microbial consortium was determined using the formula given in 3.11 and showed in Table 22.

The germination percentage of the consortium treated seeds increased from 85.7 to 92.17 from 5 to 10 days after sowing recording 12.91 per cent increase over

Table 20. Evaluation of microbial consortium on biometric characters of tomato

Sl No.	Treatments	Plant height (cm)			Days to flowering	Days to fruiting
		30 DAP	45 DAP	60 DAP		
1	T_1 - Seed treatment	37.31^{bc}	62.44^{ab}	76.59^{a}	40.74^{a}	50.07^{a}
2	T_2 - Seedling dip	40.54^{abc}	67.82^{a}	79.71^{a}	37.67^{a}	46.99^{a}
3	T_3 - Soil application at the time of planting	43.09^{a}	62.97^{ab}	74.33^{a}	41.37^{a}	50.70^{a}
4	T_4 - Seed treatment + Soil application at the time of planting	38.25^{bc}	63.61^{ab}	76.59^{a}	40.17^{a}	49.51^{a}
5	T_5 - Seed treatment + Seedling dip	44.97^{a}	65.2^{ab}	80.25^{a}	39.28^{a}	48.61^{a}
6	T_6 - Seed treatment + Seedling dip + Soil application at 45 DAP	41.5^{ab}	68.46^{a}	82.59^{a}	37.57^{a}	46.89^{a}
7	T_7 - Control (with pathogen)	36.5^{c}	61.34^{ab}	72.5^{a}	41.58^{a}	50.92^{a}
8	T_8 - Absolute control	36.57^{c}	61.47^{b}	72.06^{a}	41.87^{a}	51.19^{a}

DAP: Days after transplanting

Treatment means with same alphabets in superscript, do not differ significantly

Table 21. Reisolation of endophytes from pot culture experiment

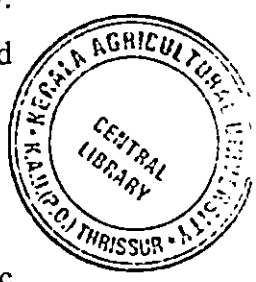
Samples	60 Days after planting			At the time of harvest		
	Fungi (cfu g^{-1})	Bacteria (cfu g^{-1})	Actinomycetes (cfu g^{-1})	Fungi (cfu g^{-1})	Bacteria (cfu g^{-1})	Actinomycetes (cfu g^{-1})
Soil	22×10^4	68×10^8	13×10^5	19×10^4	48×10^8	10×10^5
Root	14×10^1	44×10^4	6×10^1	10×10^1	30×10^4	5×10^1
Stem	$6x\ 10^1$	28×10^3	3×10^1	5×10^1	20×10^3	3×10^1

Table 22. Effect of microbial consortium on seedling vigour of tomato

Treatments	Germination percentage		Seedling length (cm)	Vigour index	Wet weight (g)	Dry weight (g)
	5 DAS	10 DAS				
With consortium	85.7	92.17	15.64	1441.69	0.658	0.067
Control	76.5	81.63	10.44	852.01	0.464	0.048
Per cent increase over control	12.03	12.91	49.86	69.21	41.81	39.58

DAS – Days after sowing

control. With respect to seeding length, consortium treated plants showed 15.64 cm against 10.44 cm in control registering 49.86 per cent increase over control. There was 69.21 per cent increased vigour index in consortium treated seedlings (1441.69) over control (852.01). Wet weight and dry weight of the treated seedlings showed 41.81 and 39.58 per cent increase over control respectively. Thus the study indicated that, seed treatment with endophytic consortium had effect in enhancing the vigour index of seedlings.



4.12. MECHANISM OF ANTAGONISM OF ENDOPHYTES

Production of volatile and nonvolatile metabolites, siderophore, salicylic acid and IAA by various isolates of endophytes were studied to know their inhibitory effect on bacterial wilt pathogen (Plate 6).

4.12.1. Production of ammonia by endophytic antagonists

The production of ammonia by the isolates was detected by the colour change of peptone water on addition of Nessler's reagent. All endophytic isolates showed positive reaction indicating ammonia production in varying level with maximum in *B. subtilis* (Plate 6a).

4.12.2. Production of HCN

HCN production by the selected endophytic isolates was tested *in vitro* following the method of picric acid assay. None of the isolates showed HCN production as no colour change of filter paper from yellow to brown/red was noticed (Plate 6b).

4.12.3. Effect of volatile metabolites of antagonistic endophytes on *R. solanacearum*

The volatile metabolites produced by the endophytic isolates showed 15.51 to cent per cent inhibition of the pathogen at fifth day of inoculation (Table 23). It is

PLATE 6

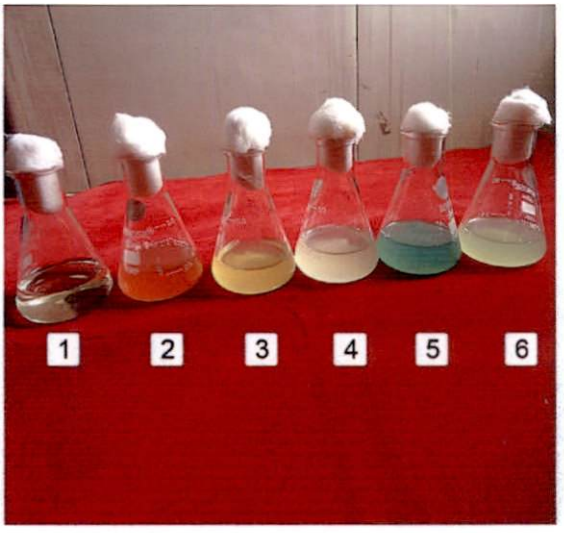
MECHANISM OF ANTAGONISM

A. Production of volatile metabolites

B. Production of non volatile metabolites

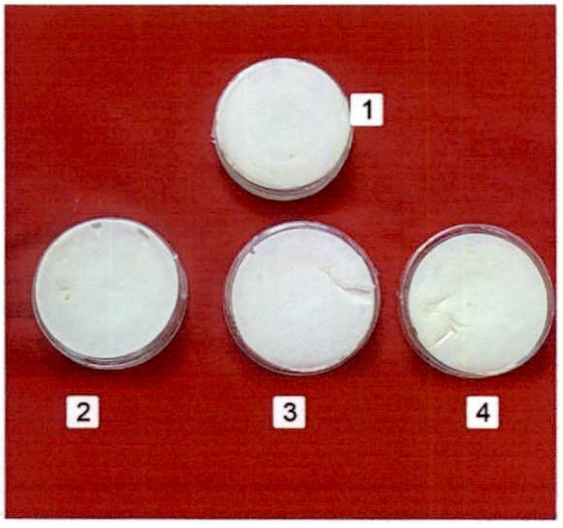
1.Control 2.*T.harzianum* 3.*T. viride*-**1**
4. *T. viride*-**2** 5. *B.subtilis* 6.*S.thermodiastaticus*

C. Production of ammonia



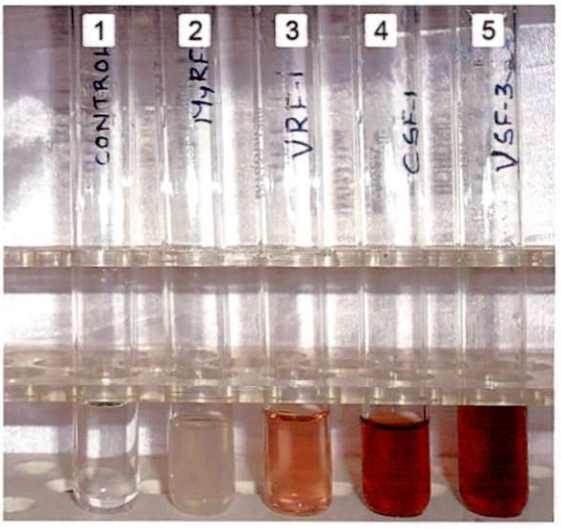
1.Control 2.*T.harzianum* 3. *T.viride*-**1**
4. *T.viride*-**2** 5. *B.subtilis* 6.*S.thermodiastaticus*

D. Production of HCN



1. Control **2.** *B.subtilis* 3. *T. viride*-**1**
4. *S.thermodiastaticus*

E. Production of IAA



1. Control 2.*T. viride*-**1** 3.*Penicillium melinii*
4. *T. viride*-**2** 5. *T.harzianum*

Table 23. Effect of volatile metabolites of endophytes on *R. solanacearum*

Sl. No.	Isolates	*Per cent inhibition
1	*B. subtilis*	100
2	*T. harzianum*-1	18.96
3	*T. viride*-1	15.51
4	*T. viride*-2	44.8
5	*S. thermodiastaticus*	31.03

* Mean of three replications

observed that, *B. subtilis* showed complete inhibition followed by *T. viride-2* (MyRF-1) with 44.8 per cent inhibition (Plate 6c & d).

4.12.4. Effect of non volatile metabolites of endophytes on *R. solanacearum*

The production of nonvolatile metabolites by endophytic isolates was studied by both culture filtrate and cellophane paper methods. It was evident from both methods that, all the selected endophytes produced nonvolatile metabolites as no growth of *R. solanacearum* was observed on culture filtrate amended media (Plate 6e).

4.12.5. Siderophore production

The selected endophytes were tested for the production of siderophore using both solid and liquid media. None of the isolates showed greenish yellow fluorescent pigments either on $FeCl_3$ amended King's B medium or in sodium succinate broth indicating the absence of siderophore production.

4.12.6. Production of IAA

Quantitative estimation of IAA produced by endophytic isolates was carried out using Salkowsky assay method (Plate 6f). The data furnished in Table 24 indicated that, all the selected endophytic organisms were found to produce varying levels of IAA ranged from 34 to 192.17 μgml^{-1} with high production by fungi as compared to bacteria and actinomycetes. The maximum amount (192.17 μgml^{-1}) was noticed with *T. harzianum*-1 followed by *T. viride*-2 (148.42 μgml^{-1}) (Fig. 10).

4.12.7. Production of salicylic acid

It is evident from Table 25 that, all selected endophytes produced salicylic acid in varying quantities ranged from 3.42 to 23.48 μgml^{-1} with maximum production in bacteria (Fig. 11). *B. subtilis* showed maximum amount

Table 24. Quantitative estimation of IAA production by endophytes

Sl. No.	Isolates	Concentration of IAA ($\mu g\ ml^{-1}$)
1	*B. subtilis*	124.25
2	*T. harzianum*-1	192.17
3	*T. viride*-1	148.42
4	*T. viride*-2	69.42
5	*S. thermodiastaticus*	10.67

Table 25. Quantitative estimation of salicylic acid production by endophytes

Sl. No	Isolates	Concentration of SA ($\mu g\ ml^{-1}$)
1	*B. subtilis*	23.48
2	*T. viride-1*	6.70
3	*T. harzianum-1*	3.65
4	*T. viride-2*	5.62
5	*S. thermodiastaticus*	3.42

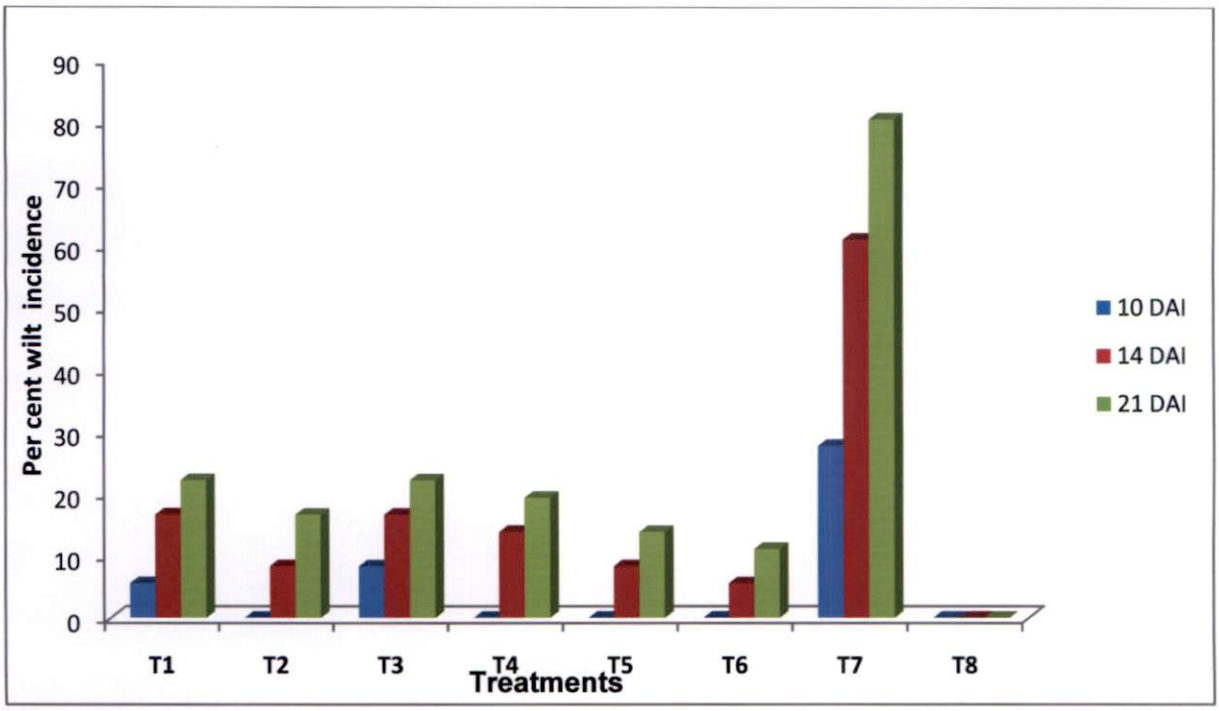


Fig. 9 Evaluation of endophytic consortium against bacterial wilt disease

T_1 -Seed treatment T_2 - Seedling dip T_3 - Soil application at the time of planting
T_4 - Seed treatment + Soil application at the time of planting T_5 - Seed treatment + Seedling dip
T_6 - Seed treatment + Seedling dip + Soil application at 45 DAP T_7 - Control (with pathogen)
T_8 - Absolute control DAI – Days after inoculation

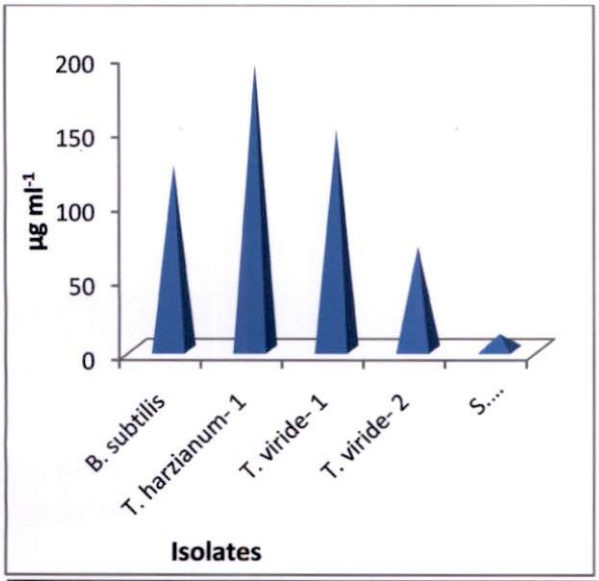


Fig. 10 Estimation of IAA production by endophytes

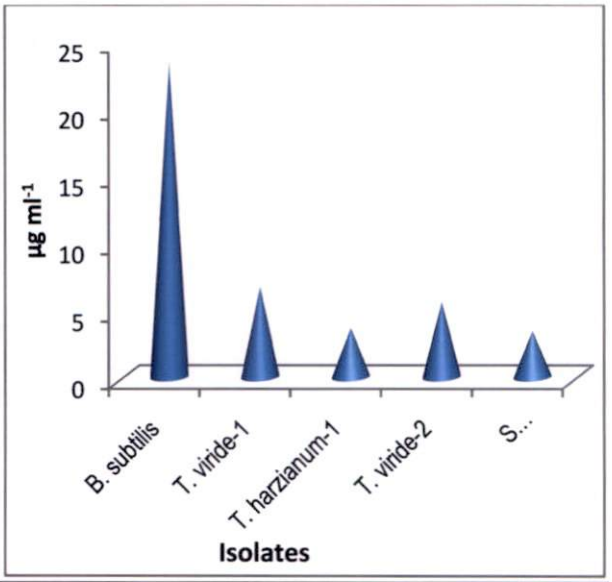


Fig. 11 Estimation of salicylic acid production by endophytes

(23.48 μgml^{-1}) and minimum production was noticed with actinomycete, *S. thermodiastaticus* (3.42 μgml^{-1}).

Summing up the findings on mechanism of antagonism, it is observed that, volatile and nonvolatile metabolites and salicylic acid had role in the antagonistic activity of the endophytes on bacterial wilt pathogen in addition to the role of IAA in promoting plant growth.

4.13. EFFECT OF SECONDARY METABOLITES OF THE SELECTED ENDOPHYTES ON *R. SOLANACEARUM*

The effect of secondary metabolites of the potential endophytes on the pathogen was studied both *in vitro* and *in vivo*.

4.13.1. *In vitro* evaluation of secondary metabolites of endophytes against *R. solanacearum*

Complete inhibition of the pathogen was observed on the medium amended with culture filtrate of the endophytes indicating the inhibitory effect of the secondary metabolites.

4.13.2. *In vivo* evaluation of secondary metabolites of the endophytes against the pathogen

Effect of secondary metabolites on *R. solanacearum* was studied under *in vivo* condition using the diluted and undiluted culture filtrate of the endophytes along with culture suspensions as comparison (Plate 7). Seedlings dipped in undiluted culture filtrate before planting showed wilting symptom in 24 to 48 h indicating phytotoxicity. Therefore, the diluted culture filtrate of 30 per cent concentration was used for the experimental purpose.

The data furnished in Table 26 revealed that, seedlings dipped first in culture filtrate/suspension then in bacterial ooze showed lowest wilt incidence and among the two, seedling dip in filtrate was the effective one. The minimum incidence was noticed with seedlings dipped first in culture filtrate of *T. viride*-2

PLATE 7. EFFECT OF SECONDARY METABOLITES OF ENDOPHYTES ON BACTERIAL WILT

Filtrate ⟶ Ooze

Ooze ⟶ Filtrate

Suspension ⟶ Ooze

Ooze ⟶ Suspension

Table 26. Effect of secondary metabolites of endophytes on bacterial wilt pathogen under *in planta* condition

Endophyte	* Per cent wilt incidence											
	7 DAI				10 DAI				14 DAI			
	Filtrate ↓ Ooze	Ooze ↓ filtrate	Suspension ↓ Ooze	Ooze ↓ Suspension	Filtrate ↓ Ooze	Ooze ↓ Filtrate	Suspension ↓ Ooze	Ooze ↓ Suspension	Filtrate ↓ Ooze	Ooze ↓ filtrate	Suspension ↓ Ooze	Ooze ↓ Suspension
T. harzianum	12.50^{b} (0.35)	27.78^{bcd} (0.55)	27.78^{bc} (0.55)	44.44^{b} (0.733)	16.67^{b} (0.42)	38.89^{bc} (0.68)	33.33^{b} (0.61)	55.56^{bc} (0.85)	22.22^{b} (0.49)	44.44^{bc} (0.73)	44.44^{b} (0.73)	61.11^{bc} (0.90)
T. viride-1	22.22^{b} (0.49)	34.92^{b} (0.63)	33.33^{b} (0.61)	47.62^{b} (0.76)	33.33^{b} (0.61)	45.24^{bc} (0.74)	38.89^{b} (0.68)	57.94^{bc} (0.87)	38.89^{b} (0.67)	50.00^{bc} (0.79)	50.00^{b} (0.79)	63.49^{b} (0.93)
T. viride-2	10.92^{b} (0.33)	19.84^{cd} (0.46)	20.64^{bc} (0.47)	27.78^{bc} (0.55)	15.68^{b} (0.39)	29.37^{c} (0.56)	25.40^{bc} (0.52)	38.89^{cd} (0.67)	19.84^{c} (0.46)	36.51^{c} (0.65)	34.92^{bc} (0.63)	44.44^{cd} (0.73)
B. subtilis	12.50^{b} (0.35)	15.08^{d} (0.40)	17.26^{c} (0.41)	16.67^{c} (0.42)	16.67^{b} (0.42)	25.40^{c} (0.52)	17.26^{c} (0.41)	27.78^{d} (0.55)	22.22^{b} (0.49)	34.92^{c} (0.63)	26.19^{c} (0.53)	38.89^{d} (0.68)
S. thermodiastaticus	20.64^{bc} (0.47)	38.89^{b} (0.68)	22.22^{bc} (0.49)	44.44^{b} (0.73)	27.78^{b} (0.55)	50.00^{b} (0.79)	30.16^{bc} (0.58)	66.67^{b} (0.97)	38.89^{b} (0.67)	61.11^{b} 0.90)	40.48^{bc} (0.69)	77.78^{b} (1.09)
Control-1 (Medium →ooze)	83.33^{a} (1.15)	83.33^{a} (1.15)	83.33^{a} (1.15)	83.33^{a} (1.15)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)
Control-2 (Ooze → medium)	88.89^{a} (1.22)	88.89^{a} (1.22)	88.89^{a} (1.22)	88.89^{a} (1.22)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)	100^{a} (1.37)

* Mean of three replications DAI – Days after inoculation

Treatment means with same alphabets in superscript do not differ significantly. Figures in parenthesis are arc-sine transformed values.

recording 10.92, 15.68 and 19.84 per cent followed by *T. harzianum*-1 and *B. subtilis*, which showed 12.5, 16.67 and 22.22 per cent at 7, 10 and 14 days after inoculation respectively. The isolates, *T. viride*-1 and *S. thermodiastaticus* were least effective, but found superior to control. Overall performance of various isolates at 14 DAI revealed that, *B. subtilis* was the most efficient one as it recorded lowest wilt incidence of 22.22, 34.92, 26.19 and 38.89 against cent per cent in control, in all the four types of treatments employed (Fig. 12). However, not much significant difference was noticed among the treatments with different isolates and were found to be on par.

In the case of seedling dip first in suspension and then in bacterial ooze, *B. subtilis* was found effective showing minimum wilt incidence at all intervals of observation and same trend was observed in seedling dip first in ooze followed by filtrate.

When the seedlings were treated first with ooze and then in culture suspension, the isolate *B. subtilis* and *T. viride*- 2 showed less incidence as compared to control. Thus the above findings clearly indicated the inhibitory effect of secondary metabolites of the endophytes on bacterial wilt pathogen.

4.14. INDUCTION OF SYSTEMIC RESISTANCE IN TOMATO BY ENDOPHYTIC MICROBIAL CONSORTIUM

The defense related compounds such as phenols, oxidative enzymes and PR proteins were estimated before and after inoculation of the pathogen and the results are summarized in Tables 27-33. It is observed from the tables that, the total and OD phenols were more in stem than the root, whereas, the activities of oxidative enzymes and PR proteins were higher in root as compared to stem. It is also noted that, in all cases, the plants treated with microbial consortium showed higher activity of the defense related compounds before the inoculation of the pathogen. Among the different treatments, plants treated with microbial consortium (T_1 and T_2) showed higher levels of phenols, oxidative enzymes and PR proteins especially in T_2, where plants treated with consortium and pathogen.

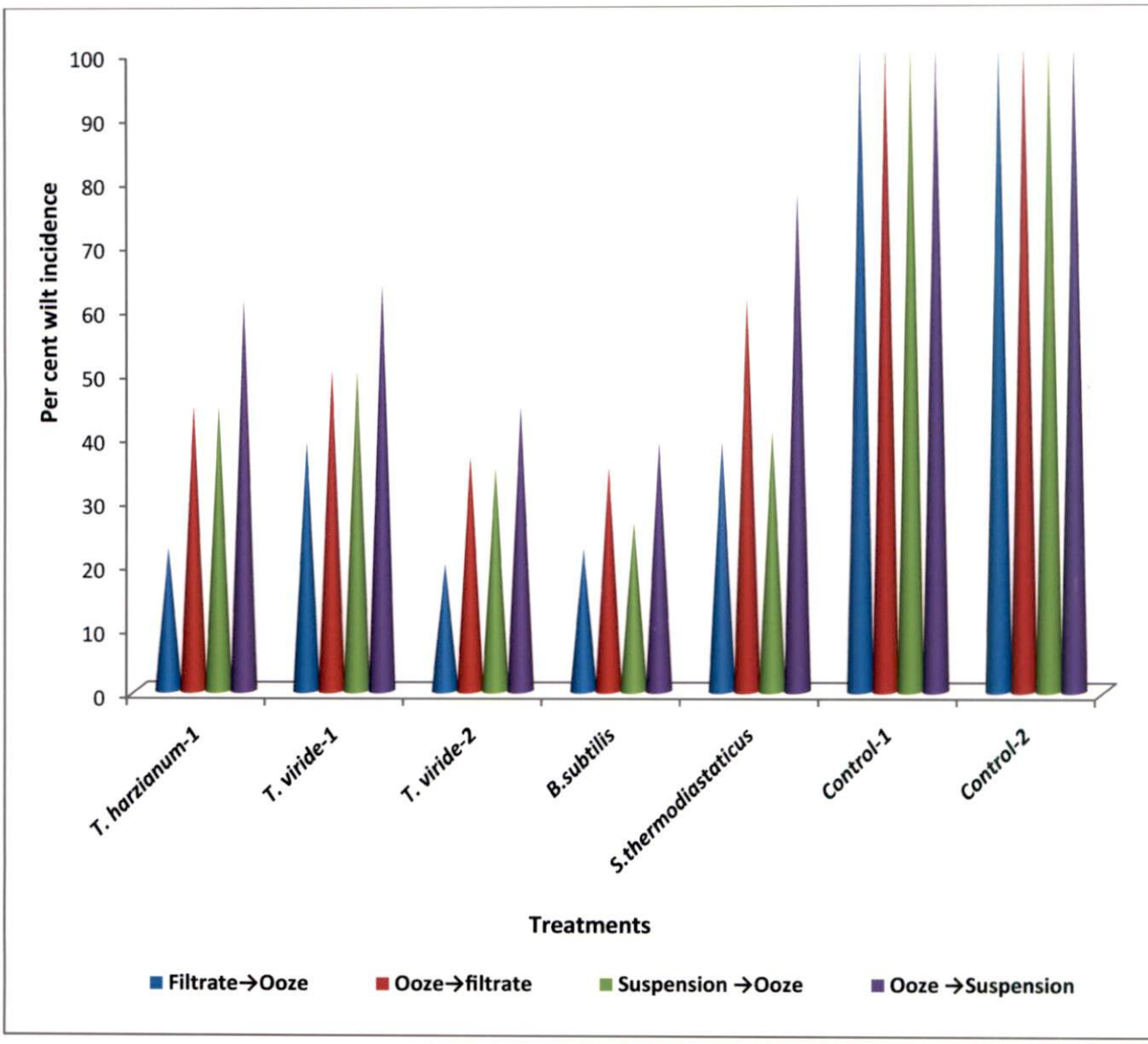


Fig. 12 Effect of secondary metabolites of endophytes on bacterial wilt under *in planta* condition

In all treatments, an initial increasing trend was observed and later decreasing/increasing pattern noticed depending upon different treatments (Plate 8).

4.14.1. Estimation of phenols

Both total and OD phenols were estimated from stem and root of tomato plants before and after inoculation of pathogen.

A. Total phenol content

Total phenol contents in stem and root of tomato plants were estimated using Folin's Ciocalteau method and the data are furnished in Table 27. The phenol content was more in stem as compared to root and varied from 2.69 to 3.21 and 2.86 to 3.64 mg g^{-1} in root and stem respectively before inoculation of the pathogen. After challenge inoculation, all treatments showed an increasing trend upto 5 DAI in both root and stem samples with maximum in T_2 (5.13 mg g^{-1}) followed by T_1 (3.35 mg g^{-1}). At 10 DAI, an increasing trend was noticed in consortium treated plants (T_1) whereas decreasing trend was observed in plants treated with consortium and pathogen (T_2) and pathogen alone (T_3). However, consortium treated plants (T_1 and T_2) showed high phenol contents compared to other treatments in both root and stem during all the intervals of observations (Fig. 13).

B. Estimation of ortho dihydroxy (OD) phenols

OD phenol contents in both root and stem, before and after inoculation of the pathogen, were estimated by Arnow's method.

It is noticed from Table 28 that, OD phenol contents before inoculation varied from 0.29 to 0.46 and 0.37 to 0.55 mg g^{-1} in root and stem respectively. At 5 DAI, all treatments showed increasing trend with highest accumulation in consortium and pathogen treated one (T_2) followed by consortium alone (T_1) in both root and stem samples, whereas treatments T_1, T_2 and T_3 showed decreasing

PLATE 8

EXPERIMENT ON INDUCED SYSTEMIC RESISTANCE

A. At the time of inoculation of the pathogen

B. Ten days after inoculation of the pathogen

Table 27. Effect of different treatments on total phenol content in tomato

Treatments	* Total phenol content (mg g^{-1} sample)					
	Root			Stem		
	0 DAI	5 DAI	10 DAI	0 DAI	5 DAI	10 DAI
T_1	3.1	3.35	3.42	3.47	3.61	3.95
T_2	3.21	5.13	4.02	3.64	5.81	5.37
T_3	2.74	3.27	2.58	2.95	3.84	2.85
T_4	2.71	2.78	2.88	2.90	3.02	3.64
T_5	2.69	2.72	2.78	2.86	3.13	3.23

* Mean of three replications DAI – Days after inoculation

T_1 – Endophytic consortium alone

T_2 - Endophytic consortium+pathogen

T_3 – Pathogen alone

T_4 –Medium alone

T_5 – Absolute control

Table 28. Effect of different treatments on OD phenol content in tomato

Treatments	* OD phenol content (mg g^{-1} sample)					
	Root			Stem		
	0 DAI	5 DAI	10 DAI	0 DAI	5 DAI	10 DAI
T_1	0.46	0.49	0.48	0.52	0.50	0.57
T_2	0.45	0.56	0.46	0.55	0.71	0.61
T_3	0.32	0.38	0.28	0.37	0.42	0.28
T_4	0.29	0.32	0.34	0.39	0.38	0.36
T_5	0.31	0.34	0.36	0.43	0.35	0.36

* Mean of three replications DAI – Days after inoculation

T_1 – Endophytic consortium alone

T_2 - Endophytic consortium+pathogen

T_3 – Pathogen alone

T_4 –Medium alone

T_5 – Absolute control

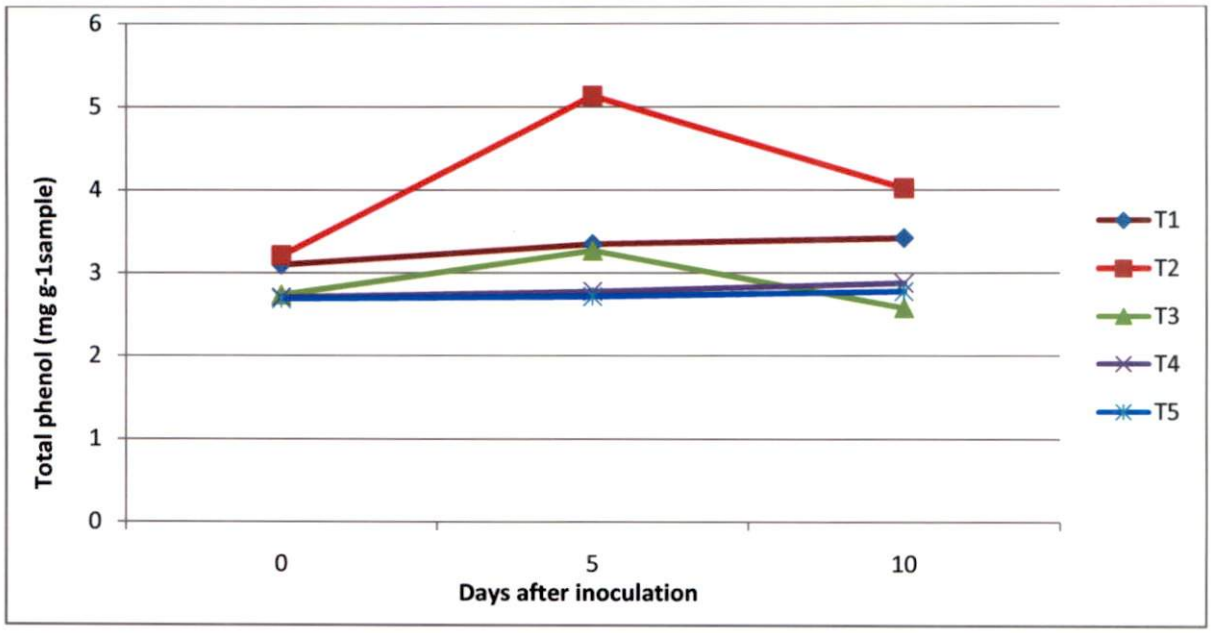


Fig. 13 Effect of different treatments on total phenol content in the roots of tomato

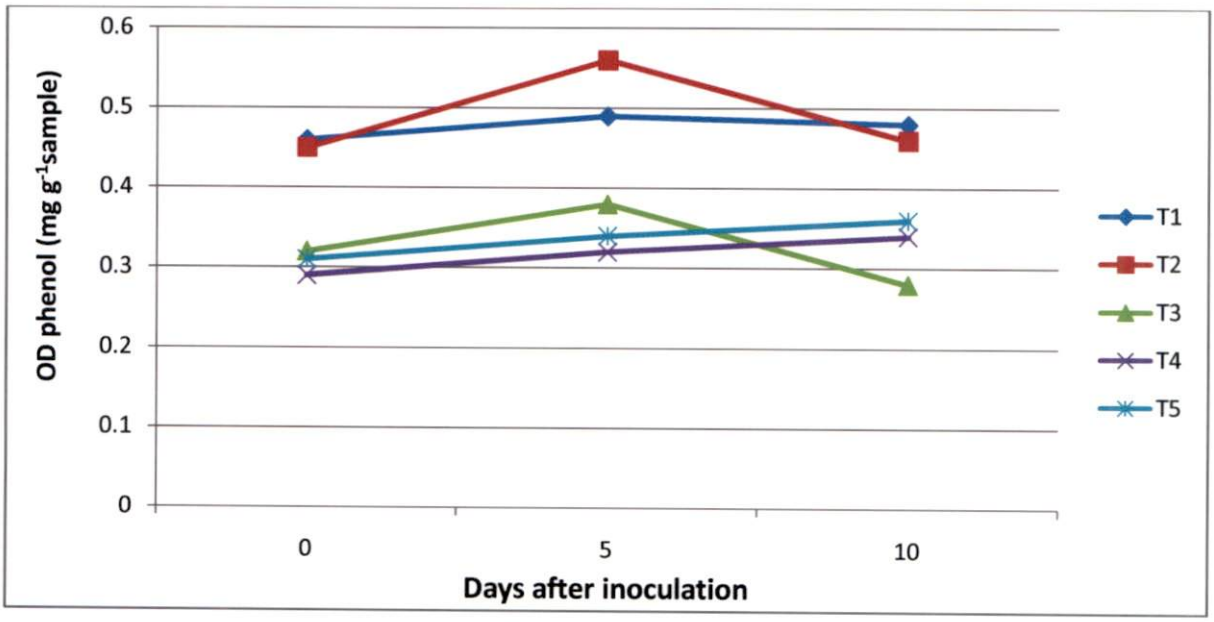


Fig. 14 Effect of different treatments on OD phenol content in the roots of tomato

T_1 – Endophytic consortium alone T_2 - Endophytic consortium+pathogen T_3 – Pathogen alone
T_4 –Medium alone T_5 – Absolute control

trend at 10 DAI. Plants treated with pathogen alone (T_3) showed less content as compared to control treatments (T_4 and T_5) (Fig. 14).

4.14.2. Assay of defence related enzymes

The activity of various defense related enzymes was studied using spectral analysis at periodical intervals. The results of the enzyme assays are presented below.

A. Peroxidase (PO) activity

The activity of PO as expressed by change in absorbance ranged from 1.06 to 1.42 $min^{-1}g^{-1}$ and 1.03 to 1.29 $min^{-1}g^{-1}$ in root and stem respectively before inoculation. The enzyme activity increased in all the treatments upto five days after inoculation with the maximum in consortium and pathogen treated root and stem (T_2) recording 2.41 and 2.23 $min^{-1}g^{-1}$ respectively followed by T_1 (consortium alone). However, at 10 DAI, a decreasing trend was observed in pathogen inoculated treatments, T_2 (consortium + pathogen) and T_3 (pathogen alone) in both root and stem samples which ranged from 1.22 to 1.96 and 1.18 to 2.02 $min^{-1}g^{-1}$ respectively with higher activity in consortium treated plants (T_1 and T_2), compared to other treatments. Any noticeable difference was observed in treatments with medium alone (T_4) and untreated control (T_5) (Table 29 & Fig. 15).

B. Polyphenol oxidase (PPO)

Before inoculation of the pathogen, PPO activity in root and stem ranged from 0.37 to 0.5 and 0.24 to 0.32 $min^{-1}g^{-1}$ respectively. On inoculation of the pathogen, enhanced activity of PPO was observed in plants treated with consortium (T_2) at 5 DAI, with maximum of 0.77 and 0.49 $min^{-1}g^{-1}$ in root and stem respectively. Though the enzyme activity was found to be declined at 10 DAI in T_1, T_2 and T_3 treatments, the consortium treated plants (T_1 and T_2) showed better activity as compared to others with maximum of 0.69 $min^{-1}g^{-1}$ (root) and 0.45 $min^{-1}g^{-1}$ (stem) in T_2 (Table 30 & Fig. 16).

C. Phenyl alanine ammonialyase (PAL)

Initially, the activity of PAL varied from 2.01 to 2.94 and 1.02 to 1.98 n mol transcinnamic acid in root and stem respectively. Same trend as in PO and PPO was observed at 5 DAI with maximum in T_2 showing 4.29 and 3.32 nmol trans cinnammic acid in root and stem respectively. Though the activity was found to be reduced at 10 DAI in all treatments except control (T_5), a drastic decline was noticed in T_2 from 4.29 to 3.06 in root and 3.32 to 2.09 n mol trans cinnammic acid in stem. In this case also, higher activity was observed in T_1 and T_2 of consortium treatments (Table 31 & Fig. 17).

4.14.3. Assay of pathogenesis related proteins (PR proteins)

The activity of major PR proteins *viz.* β-1,3-glucanase and chitinase were analyzed spectrophotometrically at periodical intervals and the results are presented below.

A. β-1,3-glucanase activity

The activity of β-1,3-glucanase was found higher in root samples as compared to stem. Before the challenge inoculation, the activity ranged from 0.48 to 0.62 and 0.16 to 0.22 glucose units released in root and stem respectively. The treatments with consortium and pathogen showed maximum activity at 5 DAI followed by T_3. The activity was found increased at 10 DAI in consortium alone treated plants (T_1) in both root and stem whereas the activity was found to be reduced in plants inoculated with pathogen (T_2 and T_3). However, the activity was higher in consortium treatments (T_1 and T_2) in both root and stem at 10 DAI (Table 32 & Fig. 18).

B. Chitinase activity

The root samples showed higher chitinase activity as compared to stem. On fifth day of inoculation, enhanced activity was observed in all treatments in root varied from 1.3 to 2.2 n mol $G_1cNAc\ min^{-1}g^{-1}$ with maximum in consortium + pathogen treatment (T_2) followed by consortium alone (T_1). Among the stem

Table 29. Effect of different treatments on peroxidase activity in tomato

Treatments	*Δ_{436} ($min^{-1}g^{-1}$ fresh tissue)					
	Root			Stem		
	0 DAI	5 DAI	10 DAI	0 DAI	5 DAI	10 DAI
T_1	1.42	1.78	1.62	1.29	1.98	1.76
T_2	1.38	2.41	1.96	1.26	2.23	2.02
T_3	1.18	1.38	1.22	1.12	1.44	1.34
T_4	1.06	1.21	1.34	1.03	1.12	1.18
T_5	1.10	1.32	1.41	1.09	1.24	1.45

Table 30. Effect of different treatments on polyphenol oxidase activity in tomato

Treatments	*Δ_{420} ($min^{-1}g^{-1}$ fresh tissue)					
	Root			Stem		
	0 DAI	5 DAI	10 DAI	0 DAI	5 DAI	10 DAI
T_1	0.47	0.65	0.63	0.29	0.38	0.36
T_2	0.50	0.77	0.69	0.32	0.49	0.45
T_3	0.45	0.47	0.36	0.27	0.32	0.30
T_4	0.42	0.39	0.41	0.24	0.22	0.26
T_5	0.37	0.40	0.42	0.24	0.27	0.29

Table 31. Effect of different treatments on phenylalanine ammonia lyase activity in tomato

Treatments	*Nmol trans cinnammic acid g^{-1} fresh tissue					
	Root			Stem		
	0 DAI	5 DAI	10 DAI	0 DAI	5 DAI	10 DAI
T_1	2.75	2.84	2.83	1.78	1.97	1.86
T_2	2.94	4.29	3.06	1.98	3.32	2.09
T_3	2.21	2.42	2.31	1.27	1.45	1.34
T_4	2.09	2.18	2.13	1.12	1.21	1.18
T_5	2.01	2.04	2.08	1.02	1.01	1.09

* Mean of three replications DAI – Days after inoculation

T_1 – Endophytic consortium alone
T_2 - Endophytic consortium+pathogen
T_3 – Pathogen alone
T_4 –Medium alone
T_5 – Absolute control

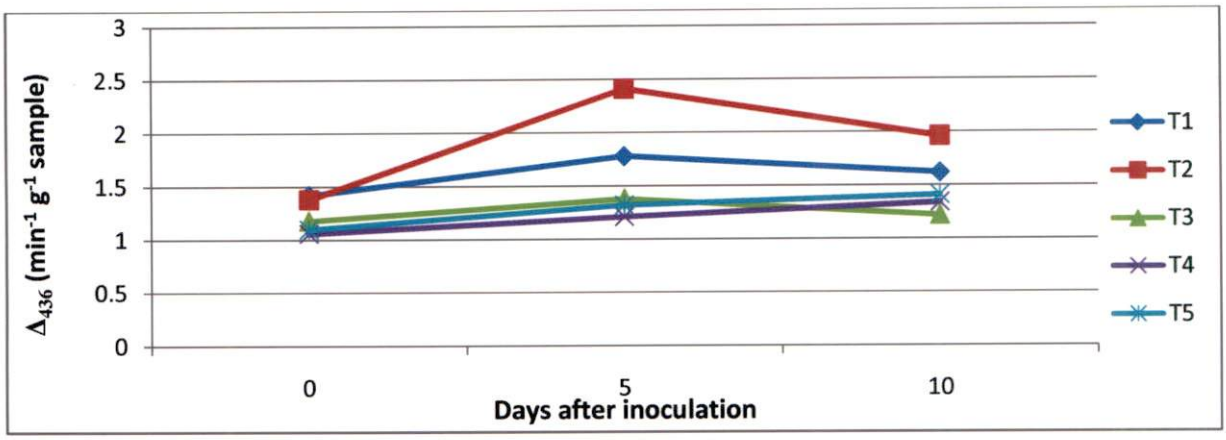


Fig. 15 Effect of different treatments on peroxidase activity in the roots of tomato

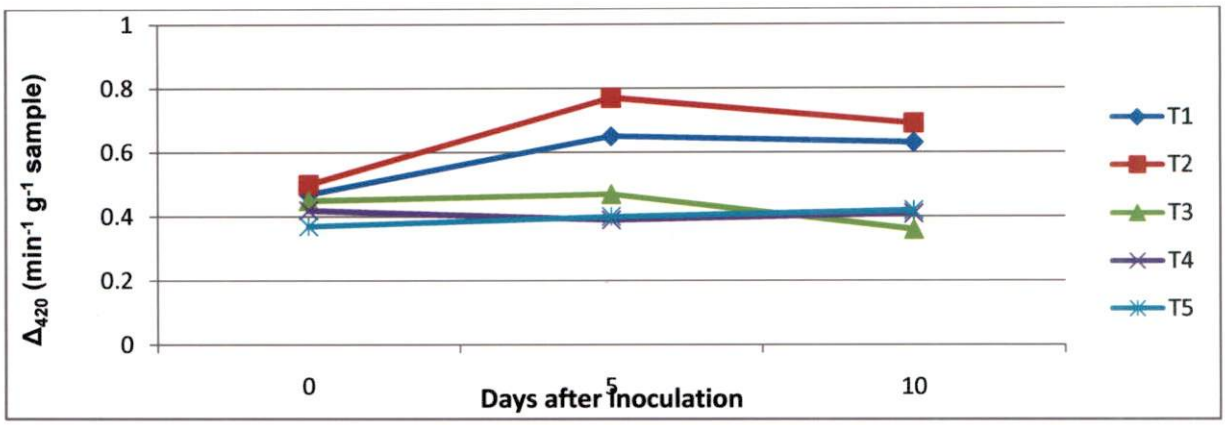


Fig. 16 Effect of different treatments on polyphenol oxidase activity in the roots of tomato

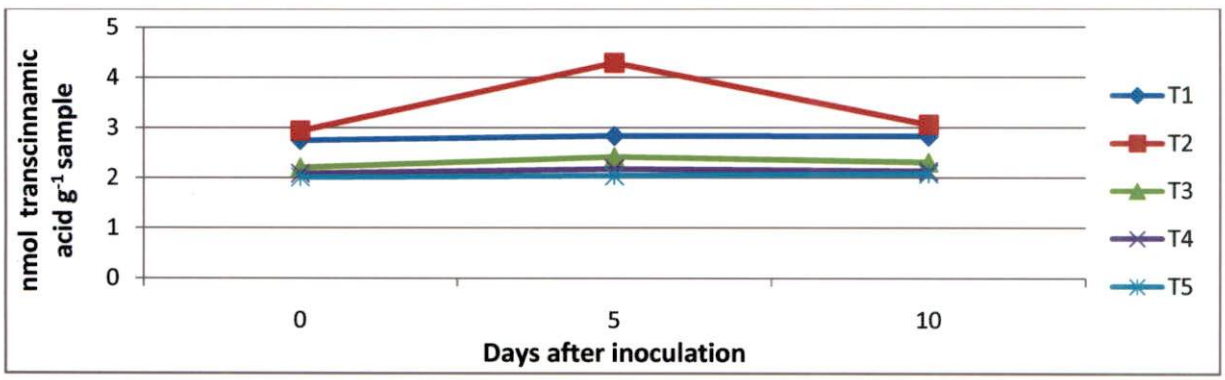


Fig. 17 Effect of different treatments on phenylalanine ammonia lyase activity in the roots of tomato

T_1 – Endophytic consortium alone T_2 - Endophytic consortium+pathogen T_3 – Pathogen alone
T_4 –Medium alone T_5 – Absolute control

Table 32. Effect of different treatments on glucanase activity of tomato

Treatments	*Glucose released (mg $min^{-1}g^{-1}$ fresh tissue)					
	Root			Stem		
	0 DAI	5 DAI	10 DAI	0 DAI	5 DAI	10 DAI
T_1	0.62	0.66	0.69	0.20	0.33	0.41
T_2	0.60	0.83	0.71	0.22	0.64	0.47
T_3	0.51	0.7	0.55	0.17	0.22	0.28
T_4	0.50	0.54	0.51	0.19	0.21	0.22
T_5	0.48	0.51	0.53	0.16	0.18	0.21

* Mean of three replications DAI – Days after inoculation

T_1 – Endophytic consortium alone T_4 –Medium alone

T_2 - Endophytic consortium+pathogen T_5 – Absolute control

T_3 –Pathogen alone

Table 33. Effect of different treatments on chitinase activity of tomato

Treatments	*Nmol G1cNAc ($min^{-1}g^{-1}$ fresh tissue)					
	Root			Stem		
	0 DAI	5 DAI	10 DAI	0 DAI	5 DAI	10 DAI
T_1	1.7	1.8	1.6	0.82	0.79	0.83
T_2	1.9	2.2	1.8	0.84	1.21	0.92
T_3	1.4	1.5	1.3	0.62	0.87	0.58
T_4	1.2	1.5	1.4	0.60	0.57	0.56
T_5	1.2	1.3	1.2	0.61	0.55	0.51

* Mean of three replications DAI – Days after inoculation

T_1 – Endophytic consortium alone T_4 –Medium alone

T_2 - Endophytic consortium+pathogen T_5 – Absolute control

T_3 –Pathogen alone

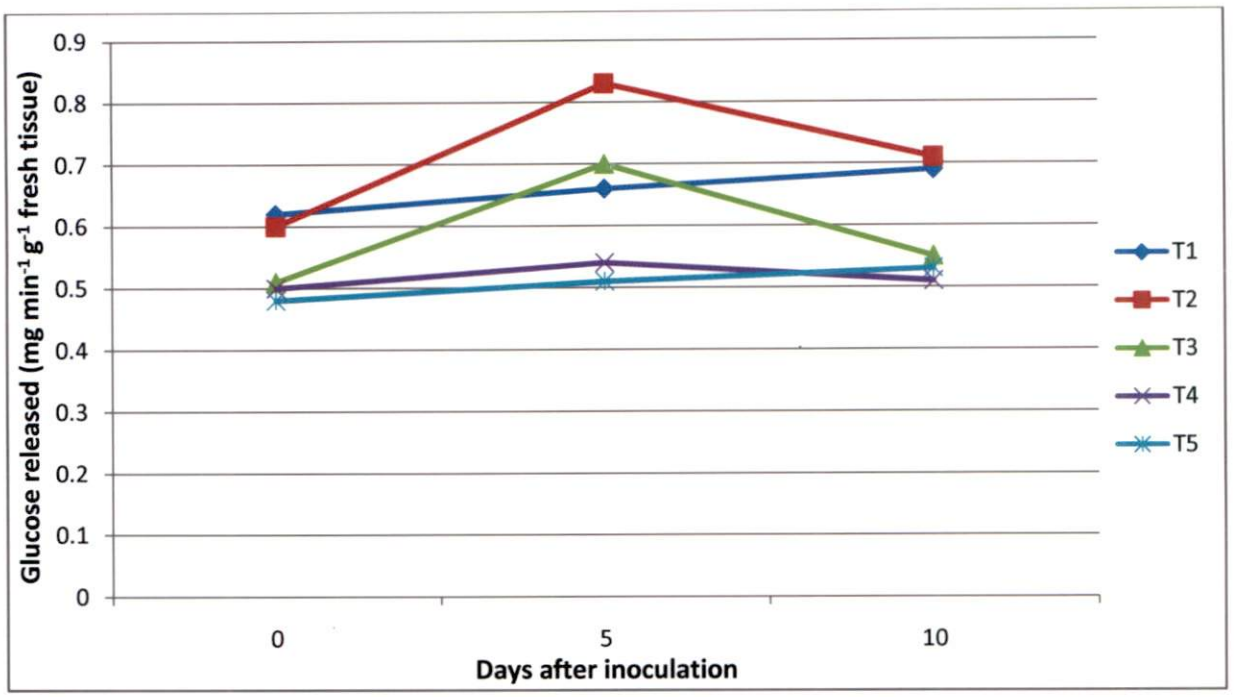


Fig. 18 Effect of different treatments on glucanase activity of tomato

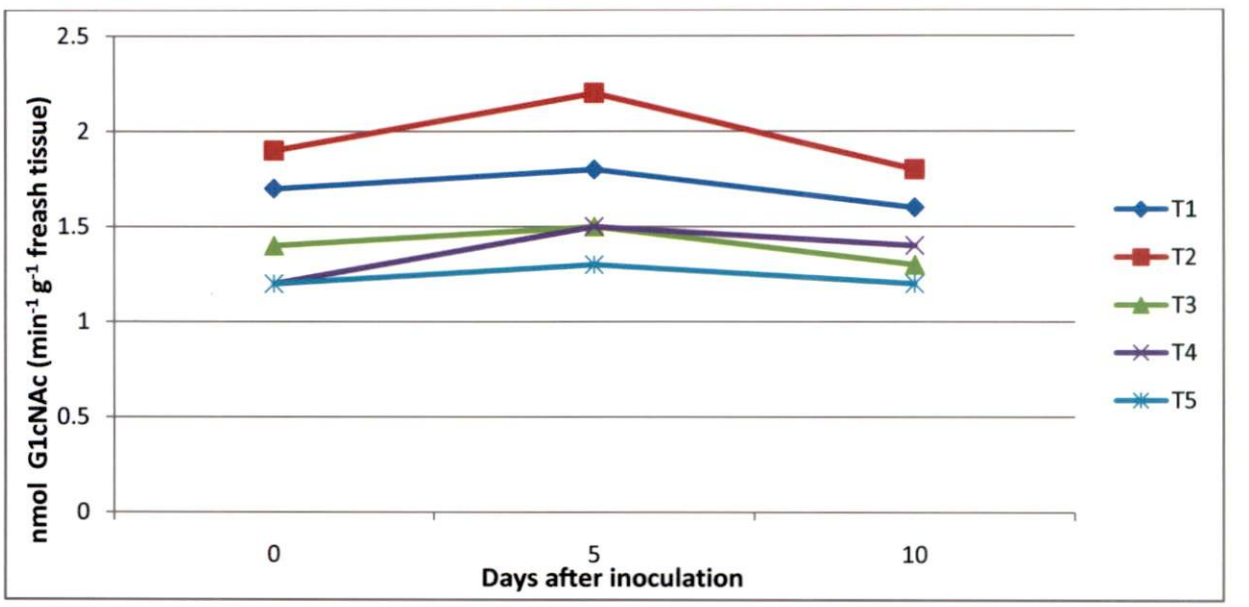


Fig. 19 Effect of different treatments on chitinase activity of tomato

T_1 – Endophytic consortium alone T_2 - Endophytic consortium+pathogen T_3 – Pathogen alone
T_4 –Medium alone T_5 – Absolute control

samples also, treatment T_2 showed highest activity and the activity reduced in all treatments at 10 DAI, however higher activity was noticed in consortium treatments (T_1 and T_2) (Table 33 & Fig. 19).

Summing up the above results, it is clearly evident that, consortium treated plants either before or after inoculation exhibited higher phenol contents, activity of oxidative enzymes and PR proteins, thus confirming the role of endophytic consortium in enhancing the defense mechanism in plants and it is more pronounced after infection.

4.15. FIELD EVALUATION OF ENDOPHYTIC CONSORTIUM AGAINST BACTERIAL WILT DISEASE

A field experiment was laid out to evaluate the performance of the endophytic consortium against bacterial wilt disease using highly susceptible variety, PKM-1, susceptible F_1 hybrid, COTH-3 and highly resistant variety, Anagha (Plate 9). The best method of application selected from the pot culture experiment was adopted which consisted of seed treatment +seeding dip +soil drenching at 45 DAP.

Observations on wilt incidence at 30, 45 and 60 DAP and biometric characters were recorded. The results of the experiments are summarized in Table 34 to 36.

4.15.1. Effect of different treatments on bacterial wilt incidence

It is observed from the data presented in Table 34 that, all treatments were superior to control for all the three varieties in all the three intervals of observations. At 30 DAP, wilt incidence was comparatively less in all treatments of three varieties which ranged from 0 to 22.22 per cent except in the control plot of highly susceptible variety (PKM-1) which recorded 47.22 per cent incidence.

In PKM-1, the disease incidence ranged from 8.33 to 20.84 per cent in different treatments against 47.22 per cent in control at 30 DAP. Lowest incidence (8.33%) was observed in plants treated with copper hydroxide @ 2g/l (T_4). A

PLATE 9

FIELD EVALUATION OF ENDOPHYTIC CONSORTIUM USING DIFFERENT VARIETIES

General field view

PKM-1

COTH-3

ANAGHA

MUKTHI

Table 34. Field evaluation of selected endophytic microbial consortium against bacterial wilt disease

Treatment	Per cent wilt incidence 30 DAP	Per cent reduction over control	Per cent wilt incidence 45 DAP	Per cent reduction over control	Per cent wilt incidence 60 DAP	Per cent reduction over control
V_1T_1+	13.89[a] (0.381)	70.58	54.17[a] (0.828)	44.28	58.33[a] (0.869)	40.85
V_1T_2	16.67[a] (0.399)	64.70	72.22[ab] (1.036)	25.71	83.33[bc] (1.157)	15.49
V_1T_3	20.84[a] (0.473)	55.88	84.72[ab] (1.175)	12.86	87.50[bc] (1.213)	11.27
V_1T_4	8.33[a] (0.287)	82.35	52.78[a] (0.817)	45.71	66.67[ab] (0.979)	32.39
V_1T_5	47.22[b] (0.757)	--	97.22[b] (1.365)	--	98.61[c] (1.365)	--
CD(0.05)	**0.194**		**0.364**		**0.274**	
V_2T_1+	6.94[a] (0.277)	68.75	34.72[ab] (0.628)	35.90	36.11[a] (0.644)	46.94
V_2T_2	13.89[ab] (0.381)	37.50	36.11[ab] (0.644)	33.34	48.61[a] (0.772)	28.57
V_2T_3	8.33[a] (0.277)	62.50	43.06[bc] (0.716)	20.51	47.22[a] (0.758)	30.61
V_2T_4	6.94[a] (0.286)	68.75	20.83[a] (0.473)	61.54	41.67[a] (0.701)	38.77
V_2T_5	22.22[b] (0.486)	--	54.17[c] (0.829)	--	68.06[b] (0.970)	--
CD(0.05)	**0.170**		**0.152**		**0.152**	
V_3T_1++	1.39 (1.192)	49.98	2.78 (1.462)	50.02	2.78 (1.462)	50.02
V_3T_2	0.00 (0.707)	100.00	4.17 (1.947)	25.03	4.17 (1.947)	25.03
V_3T_3	1.39 (1.192)	49.98	4.17 (1.947)	25.03	4.17 (1.947)	25.03
V_3T_4	1.39 (1.192)	49.98	1.39 (1.192)	75.01	1.39 (1.192)	75.01
V_3T_5	2.78 (1.462)	--	5.56 (2.431)	--	5.56 (2.431)	--
CD(0.05)	**0.170**		**0.152**		**0.152**	

+ Figures in paranthesis are Arc sine transformed values

++ Figures in paranthesis are square root transformed values

V1 – PKM-1 T1- Endophytic consortium T4 –Copper hydroxide 2g/l

V2 – COTH-3 T2 – Rhizospheric consortium T5 – Control

V3 - Anagha T3 – *Pseudomonas fluorescens*

drastic increase was noticed at 45 DAP which varied from 52.78 to 84.72 per cent with minimum (52.78%) in copper hydroxide (T_4) against 97.22 per cent in control (T_5). However, at 60 DAP, no significant increase was noticed in various treatments and the minimum incidence (58.33%) was observed in treatment with endophytic consortium (T_1) recording 40.85 per cent reduction over control.

In susceptible hybrid variety (COTH-3), at 30 DAP, both endophytic consortium (T_1) and copper hydroxide (T_4) treatments recorded lowest incidence of 6.94 per cent against 22.22 per cent in control (T_5). At 45 DAP, minimum incidence (20.83 %) was noticed in T_4 (copper hydroxide) with 61.54 per cent reduction followed by T_1 (endophytic consortium) showing 34.72 per cent incidence and was on par with T_2 (rhizosphere consortium). However, no significant difference was observed among the treatments at 60 DAP and all treatments were on par. Treatment with endophytic consortium (T_1) showed lowest incidence of 36.11 per cent with 46.94 per cent reduction over control. Moreover, this treatment showed only slight increase in infection from 45th to 60th day of planting whereas copper hydroxide (T_4) showed drastic increase in incidence from 20.83 to 41.67 per cent.

In resistant variety Anagha, the wilt incidence was comparatively very less, hence no significant difference was noticed among the treatments in all the three intervals of observations. At 30 DAP, plants treated with rhizospheric consortium were free of wilt disease and other treatments also showed less incidence of 1.39 per cent against 2.78 per cent in control. At 45 DAP, slight increase was noticed in all treatments except T_4 (copper hydroxide 2g/l) recording lowest incidence of 1.39 per cent with 75.01 per cent reduction over control which is closely followed by T_1 (Endophytic consortium) with 2.78 per cent against 5.56 per cent in control. Same trend was observed at 60 DAP, as no disease progression was noticed in any of the treatments (Plate 10 & Fig. 20).

In moderately resistant variety Mukthi, drastic reduction in wilt incidence was observed with the application of endophytic consortium recording 16.67,

19.44, 23.33 against 36.11, 42.78, 49.44 per cent in control at 30, 45 and 60 days after planting respectively and also recorded 52.81 per cent efficiency over control at 60 DAP (Table 36 & Fig. 21).

4.15.2. Effect of treatments on biometric characters of tomato

Biometric characters such as plant height, days to flowering, days to first harvest, number of fruits per plant, average weight of fruits, per plant yield and yield per plot were recorded and presented in Table 35.

In all the three varieties, significant difference was noticed with the different treatments compared to control with respect to plant height, with maximum of 74.99, 86.87 and 81.33 cm in treatment with endophytic consortium (T_1) against 61.02, 77.03 and 70.99 cm in control, in PKM-1, Anagha and COTH-3 respectively.

No significant difference was noticed among the treatments with respect to days to flowering and days to first harvest in all the three varieties. Among the treatments, early flowering and fruit maturity were observed with application of endophytic consortium (T_1).

Among the yield parameters, no significant difference was noticed among the treatments with respect to number of fruits per plant and average fruit weight and it is also noted that application of endophytic consortium had effect on increasing the number and weight of fruits in all the three varieties.

In case of per plant yield, all treatments were significantly superior to control in all the varieties and maximum was observed in consortium treatment (T_1) with 310.5 g in PKM-1 and 563.8 g in COTH-3 against 246.3 g and 382.55 g in control respectively, whereas, in Anagha, treatment with copper hydroxide (T_4) recorded maximum per plant yield with 565.73 g followed by treatment with consortium treatment (T_1) with 556.97 g.

All treatments were significantly superior to control in all the varieties with respect to yield per plot. The treatment with endophytic consortium (T_1)

Table 35. Effect of selected endophytic consortium on biometric characters of tomato

Treatment	Plant height (cm)	Days to flowering (DAP)	Days to first harvest (DAP)	Average number of fruits / plant	Average weight of fruits (g)	Per plant yield (g)	Yield per plot (kg/8.64 m^2)
V_1T_1	74.99[a]	39.60[a]	72.48[a]	10.97	29.20	310.5[a]	2.67[a]
V_1T_2	72.81[a]	40.04[a]	73.69[a]	10.20	31.25	285.2[ab]	1.63[ab]
V_1T_3	73.6[a]	41.75[b]	74.08[a]	9.58	30.18	279.5[ab]	0.87[ab]
V_1T_4	70.40[a]	40.02[a]	74.90[a]	11.12	26.90	308.7[a]	1.92[a]
V_1T_5	61.02[b]	42.55[b]	78.96[b]	9.00	26.30	246.3[b]	0.25[b]
CD (0.05)	**4.756**	**1.34**	**2.768**	**NS**	**NS**	**7.353**	**1.52**
V_2T_1	81.33[a]	36.79[a]	70.13[a]	17.04	33.72	563.80[a]	8.62[a]
V_2T_2	79.79[ab]	39.05[ab]	71.03[a]	15.67	31.13	395.15[bc]	5.53[bc]
V_2T_3	78.31[ab]	38.66[ab]	71.47[a]	16.80	28.47	476.23[b]	5.9[b]
V_2T_4	74.39[ab]	39.37[ab]	70.44[a]	13.80	29.03	491.82[ab]	6.0[ab]
V_2T_5	70.99[b]	40.10[b]	75.70[b]	13.57	28.78	382.55[c]	3.25[c]
CD (0.05)	**7.891**	**2.295**	**3.445**	**NS**	**NS**	**8.238**	**3.15**
V_3T_1	86.87[a]	35.00[a]	69.00[ab]	19.80	29.12	556.97[a]	12.50[a]
V_3T_2	83.00[ab]	36.90[b]	70.23[b]	19.67	28.82	490.20[b]	10.42[b]
V_3T_3	80.87[ab]	37.03[b]	69.05[ab]	18.57	29.14	534.38[ab]	11.33[ab]
V_3T_4	78.55[b]	36.03[ab]	68.66[a]	18.40	26.10	565.73[a]	12.98[a]
V_3T_5	77.03[b]	37.30[b]	71.97[c]	15.38	24.94	394.54[c]	10.25[c]
CD (0.05)	**7.315**	**1.158**	**1.147**	**NS**	**NS**	**10.258**	**1.659**

V_1 – PKM-1
V_2 – COTH-3
V_3- Anagha

T_1- Endophytic consortium
T_2 – Rhizosphere consortium
T_3 – *Pseudomonas fluorescens*

T_4 – 0.2% Copper hydroxide
T_5 – Control

PLATE 10

EFFECT OF ENDOPHYTIC CONSORTIUM ON BACTERIAL WILT IN TOMATO

Treated plot

Control

A. PKM -1

Treated plot

Control

B. COTH - 3

Treated plot

Control

C.ANAGHA

EFFECT OF ENDOPHYTIC CONSORTIUM ON BIOMETRIC CHARACTERS OF TOMAT

Treated

Control

A. PKM -1

Treated

Control

B. COTH - 3

Treated

Control

C. ANAGHA

recorded significantly higher yield of 2.67 kg and 8.62 kg/8.64 m^2 against 0.25 kg and 3.25 kg/8.64 m^2 in control in varieties PKM-1 and COTH-3 respectively. In variety Anagha, T_4 (copper hydroxide) recorded maximum yield of 12.98 kg/ 8.64 m^2 which was on par with T_1– endophytic consortium (12.5 kg/8.64 m^2) against 10.25 kg/8.64 m^2 in control (Plate 11).

In moderately resistant variety Mukthi, endophytic consortium treated plants showed maximum plant height, early flowering, fruit maturity and yield compared to control. The yield per plot was three times higher than the control plot recording 6.38 kg against 2.08 kg/8.64 m^2 (Table 37).

Summing up the findings of the field experiments, it is observed that, the application of microbial consortium could enhance the resistance considerably in highly susceptible, susceptible and moderately resistant varieties and to some extent in resistant ones and also promoted the plant growth, thereby increased the yield. Thus it revealed that, the microbial consortium had the ability to enhance the defense mechanism in tomato against the bacterial wilt pathogen.

Table 36. Effect of endophytic consortium on bacterial wilt in moderately resistant variety (Mukthi)

Treatments	Per cent wilt incidence					
	30 DAP	Per cent efficiency over control	45 DAP	Per cent efficiency over control	60 DAP	Per cent efficiency over control
Consortium treated	16.67	53.84	19.44	63.17	23.33	52.81
Control	36.11	-	42.78	-	49.44	-

DAP: Days after planting

Table 37. Effect of endophytic consortium on biometric characters of Mukthi

Treatments	Plant height (cm)	Days to flowering	Days to harvest	Yield/plant (g)	Yield/8.64m^2 (kg)
Consortium treated	58.4	50.5	81	623.5	6.38
Control	33.85	54.5	84.5	283	2.075

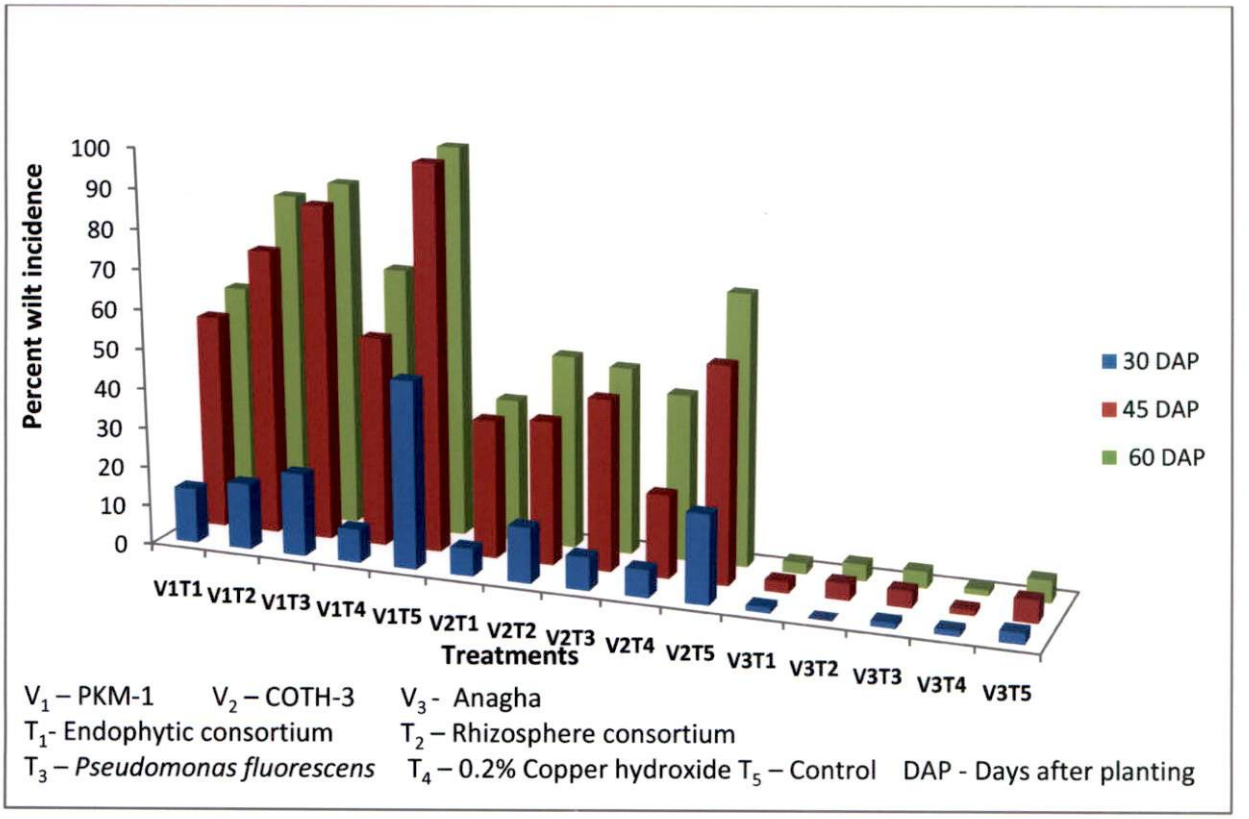


Fig. 20 Field evaluation of endophytic consortium against bacterial wilt of tomato

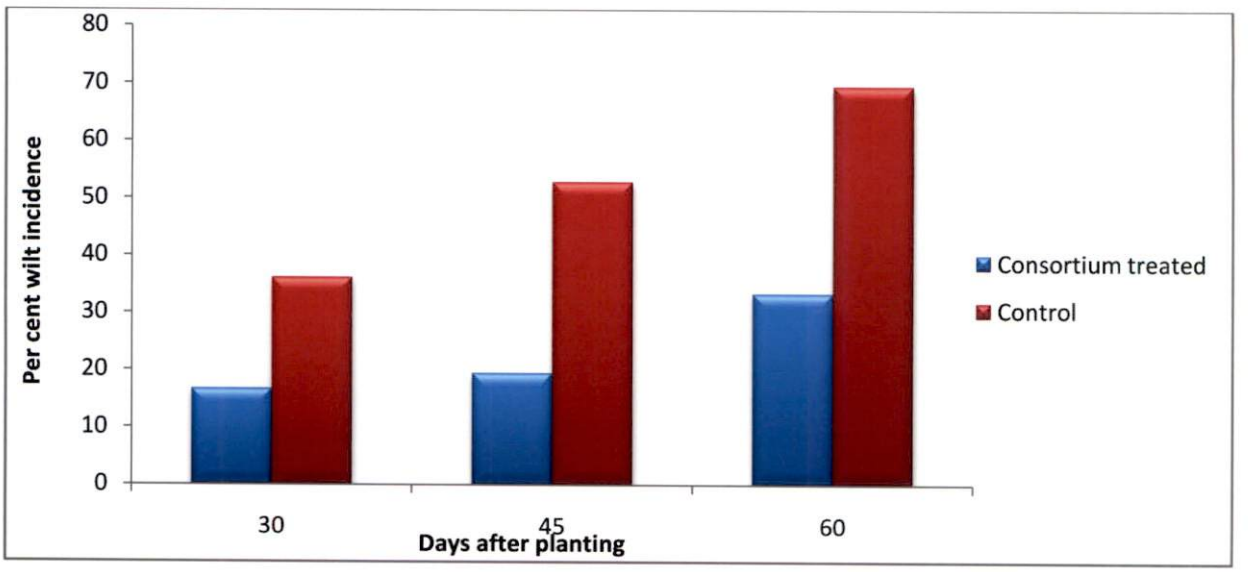


Fig. 21 Field evaluation of endophytic consortium against bacterial wilt in Mukthi variety

Discussion

5. DISCUSSION

Tomato is one of the most widely grown vegetables in the world. The high demand for tomato makes it a high value crop that can generate good income for farmers. Bacterial wilt caused by *Ralstonia solanacearum* is one of the major diseases in tomato and has posed a serious threat to tomato cultivation especially in warm humid tropics. Although it is difficult to estimate total economic losses that cause directly or indirectly by bacterial wilt, it ranks one of the most important plant diseases in the entire world (Gnanamanickam, 2006). The disease has provided many enigmas for scientists working on tomato.

R. solanacearum is a genetically diverse soil borne pathogen with a wide host range and is difficult to control the disease once it established in the field. Although application of chemicals may reduce the incidence of the bacterial diseases, it cannot be continued as long term solution because of many ecological problems as well as the chance of development of resistant strains of the pathogen. Breeding for host resistance is one of the economical and practical ways to tackle this disease and has provided some substantial success in Kerala. But it is also found fluctuating due to the extreme variability of the pathogen and aggressiveness of bacterial isolates from different locations. So alternate control measure such as biological control can be a potential mode to manage the disease. Biocontrol agents isolated from rhizosphere and phylloplane have been extensively studied for the management of plant diseases. But their success is limited because of their dependence on environmental conditions and competition for colonization of ecological niches. These problems can be overcome to a great extent by the use of endophytic antagonists since the internal habitat ensures supply of nutrients and protects from competition with other microbes (Manjula *et al.*, 2002). Hence the possibility of using endophytes as ecologically sustainable bioagents against the plant diseases has been explored in recent years.

Generally, the application of single antagonist leads to inconsistent performance. Recently, a greater thrust is being given for the development of biological consortium since it consists of microbes with different biochemical and physiological capabilities, which permit interaction among themselves, and will lead to the establishment of a stable and effective microbial community. Moreover, it will provide better management of diseases by the way of synergistic effect and multiple modes of action. Interestingly, several researchers have observed increased plant growth and improved disease control using microbial consortia comprising of various biocontrol organisms such as *Trichoderma*, *Pseudomonas*, *Bacillus* spp. etc in wheat, chickpea, tomato, chilli, black pepper, cardamom, ginger, Arabidopsis and pigeon pea (Duffy *et al.*, 1996; Sarma *et al.*, 2000; Rudresh *et al.*, 2005; Mathew, 2008; Kannan and Surender, 2009; Srivastava *et al.*, 2010). Studies have been conducted on the management of bacterial wilt of chilli using various rhizospheric consortia (Mathew, 2008). A perusal of literature revealed that, no attempts have been made so far for the management of bacterial wilt of tomato using endophytic microbial consortium. Moreover, endophytes are known to bring about disease suppression by various modes of action especially the induced systemic resistance (Barka *et al.*, 2002). Hence it is pertinent to have a detailed workup on the use of endophytes along with their consortial effect as a possible management strategy against bacterial wilt diseases. Thus, being the first attempt, the present investigation is focused to harness the potential effect of endophytic microbial communities on the management of bacterial wilt of tomato caused by *R. solanacearum*.

In order to study the diversity and distribution of endophytes in tomato, plant samples were collected from 16 different locations representing north, central and south Kerala and the endophytes were isolated from both stem and root of tomato plants. As a preliminary experiment, the dilution factors for the isolation of different endophytic organisms were standardised with Vellanikkara sample and 10^{-3} and 10^{-4} dilutions were found to be ideal for the isolation of endophytic bacteria from stem

and root respectively. Likewise, 10^{-2} was standardized as dilution factor for isolating fungi whereas 10^{-1} for actinomycetes from both stem and root. However, Nawangsih *et al.* (2011) could isolate endophytic bacteria with 10^{-5} dilution from the stem and Nandhini *et al.* (2012) isolated with dilution of 10^{-10} from the roots and stem of tomato. Kurian (2011) also used higher dilutions of 10^{-7}, 10^{-4} and 10^{-5} for the isolation of endophytic bacteria, fungi and actinomycetes respectively from cacao root and shoot. The dilution factor may vary with plant species. Similarly, Mathew (2007), Uppala (2007), and Balan (2009) used 10^{-2} dilution for the isolation of endophytic bacteria and fungi from stem and root of black pepper, amaranth and anthurium respectively. Majority of the reports on isolation of endophytic actinomycetes showed 10^{-1} dilution being the ideal for their isolation (Cao *et al.*, 2005; Tan *et al.*, 2006; Sreeja, 2011).

The isolation of endophytes were done on different media *viz.* bacteria on nutrient agar and King's B, fungi on Martin's Rose Bengal Agar and Trichoderma Selective Medium, and actinomycetes on Kenknight's Agar Medium. This helped in selecting appropriate colonies of each group of organisms separately from a large population. According to Lodge *et al.* (1996), quantitative surveys of endophyte colonization patterns may be sensitive to leaf size, age, methodology and growing medium. Hence, the use of different media with varied nutrient contents in isolation was helpful in getting more diverse group of organisms.

Quantitative estimation of endophytic microorganisms in tomato from different locations revealed that, the bacterial endophytes formed the major population compared to fungi and actinomycetes. This result is in accordance with earlier reports by Kloepper *et al.* (1980), Fisher *et al.* (1992), Uppala (2007), Balan (2009) and Kurian (2011). Endophytes originate from rhizosphere or phyllosphere and they enter the endosphere mainly through natural openings (Ryan *et al.*, 2008). Since major share of microbial population of rhizosphere or phyllosphere is contributed by bacteria, it is reasonable to expect more bacteria in the endosphere

also. It is similar to that observed by the earlier workers. It was also noticed that, root portion yielded high population of endophytes compared to stem which is in agreement with the findings of Tripathi *et al.* (2006), Mathew (2006b), Rajendran *et al.* (2006), Balan (2009) and Kurian (2011). Yang *et al.* (2011) recorded 72 endophytic bacteria from tomato including 45 from stem and 27 from leaves. Patel *et al.* (2012) isolated 18 endophytic bacteria from root and stem of tomato plants collected from different regions of Gujarat. The present study yielded 79 bacteria, 68 fungi and seven actinomycetes from root and stem of tomato plants collected from different parts of Kerala.

Variation in the population of endophytes was noticed among the samples collected from different locations. High fungal population was observed in both root and stem samples collected from Kumarakom, whereas bacteria was more in root samples from Alappuzha and the population of actinomycetes was maximum in Panniyur, Ozhalapathy and Amburi root samples. Since the endophytic population is influenced by external factors (Wilson and Carroll, 1994), samples collected from different agroclimatic locations resulted in the variation of endophytic population. This is in line with the observations of Uppala (2007), Balan (2009), Sreeja (2011) and Kurian (2011) who also noticed variation in the endophytic population in the samples collected from different locations of Kerala.

In vitro studies are useful for identifying likely candidates for biocontrol and for understanding the mechanisms by which they inhibit pathogens (Mejia *et al.*, 2008). Hence in this study, endophytic isolates obtained were screened for *in vitro* inhibitory effect on the bacterial wilt pathogen, *R. solanacearum*. Of the 79 endophytic bacteria, 31 were able to exert antagonism with 12.78 to 53.89 per cent inhibition with maximum exhibited by EkRB-1, a bacterial endophyte from Ernakulam. Sturz *et al.* (1998) isolated endophytic bacteria from potato and clover of which 74 per cent showed varying levels of *in vitro* antibiosis to the clover and potato pathogens. Mathew (2006b) also observed the inhibitory effect of endophytic bacteria

isolated from black pepper on *R. solanacearum* of chilli. Nawangsih *et al.* (2011) reported the antagonistic effect of 17 endophytic bacteria isolated from tomato stem on the pathogen, *R. solanacearum*. Feng *et al.* (2013) also reported that, out of 41 endophytic bacterial isolates obtained from tomato, only six were found to have *in vitro* antagonistic property against *R. solanacearum*. Similar findings have been recorded by Purnawati *et al.* (2014) who also noticed the antagonistic activity of the bacterial endophytes of tomato against *R. solanacearum*.

In the screening of 68 fungal endophytes, 27 showed antagonism ranged from 11.67 to 66.67 per cent inhibition of which ARF-2, a fungal endophyte from Alappuzha showed maximum antagonistic activity on the bacterial wilt pathogen. Many workers have reported the antagonistic ability of endophytic fungi against different pathogens. Haiyan *et al.* (2005) observed that, of the 130 endophytic fungi isolated from chinese medicinal plants evaluated, only 30 per cent exhibited antagonistic activity. Similarly, Mathew (2006b) also observed *in vitro* antagonistic activity of endophytic *Trichoderma* spp. against *R. solanacearum* of chilli. Kim *et al.* (2007) also reported that, endophytic fungi isolated from vegetable plants showed *in vitro* antagonism against *Pythium ultimum*, *P. infestans* and *P. capsici*.

In vitro evaluation of seven endophytic actinomycetes showed varying level of antagonism ranging from 16.67 to 27.22 per cent inhibition with maximum by Ozhalapathy isolate, ORA-1. The antagonistic effect of endophytic actinomycetes against *R. solanacearum* has already been reported by earlier workers. Moura *et al.* (1998) observed that, endophytic actinomycetes isolated from the root tissues of tomato showed cent per cent inhibition of the pathogen. Moura and de-Romero (1999) also noted high inhibitory effect of endophytic actinomycetes isolated from various hosts against *R. solanacearum*. This study also supports the findings of Sreeja (2011) who recorded 8.14 - 29.25 per cent inhibition of *R. solanacearum* with the endophytic actinomycetes of tomato from various locations of Kerala.

The ability of microorganisms to inhibit the pathogen under *in vitro* condition does not necessarily imply the same ability *in vivo*. Hence, it has become pertinent to evaluate the efficiency of selected endophytes under *in planta* condition. Among the endophytic isolates, 16 fungi, 12 bacteria and four actinomycetes which exhibited better antagonism in the *in vitro* experiment, were evaluated for their performance in plants. *In planta* evaluation showed marked variation in the level of disease reduction brought about by these isolates and five bacteria, eight fungi and two actinomycetes which showed higher efficiency, recording more than 50 per cent reduction over control were selected. There are number of ways by which antagonists suppress the growth of pathogen of which production of volatile metabolites, lytic enzymes, induction of host resistance and antagonistic proteins are important. Similarly, Mathew (2006b) and Sreeja (2011) also studied the efficacy of endophytic fungi, bacteria, and actinomycetes against *R. solanacearum* of chilli and tomato under *in planta* condition.

Summing up the findings of *in vitro* and *in planta* experiments, it is observed that, the isolates which showed high antagonistic activity under *in vitro* condition were not that effective in *in planta* condition except the bacterial isolate EkRB-1, fungal isolate MyRF-1, and actinomycete ORA-1, which performed well under both conditions. The lack of correlation between *in vitro* and *in vivo* effectiveness of biological control had already been observed by Ran *et al.* (2005) who also reported that, fluorescent pseudomonads sometimes succeeded as a biocontrol agent *in vitro* or under controlled conditions but failed under pot and field conditions.

It is likely that, most cases of naturally occurring biological control results from mixtures of antagonists, rather than from high population of a single antagonist. Mixtures of antagonists are considered to account for protection in disease suppressive soils. Combinations of biocontrol agents for plant diseases include mixtures of fungi and mixtures of fungi and bacteria. Most of the reports on bioconsortia showed that, combinations of antagonists resulted in improved

biocontrol. However, there are also reports of combinations of bioagents that do not result in improved suppression of disease compared with the individual antagonist. Incompatibility of the coinoculants can arise because biocontrol agents may also inhibit each other as well as the target pathogen. Thus an important prerequisite for successful development of microbial consortia appears to be the compatibility of the coinoculated microorganisms. Therefore, the endophytes selected from *in planta* experiment were subjected to mutual compatibility test for the development of efficient microbial consortia. Fifteen endophytes including eight fungi, five bacteria, and two actinomycetes were tested *in vitro* for their compatibility. The interactions among the eight fungal endophytes showed observations like heavy sporulation at the meeting point, diffusion of metabolite, intermingling of hyphae, yellow pigmented band at the interaction point, clear demarcation at the meeting point, and dark green spores turned olive green. Similar interactions were observed between endophytic *Trichoderma* isolates of black pepper by Mathew (2007). Among the eight fungi tested, three were noncompatible of which one showed suppression of growth and two exhibited overgrowth mechanism. Likewise, among the five bacteria, two isolates were incompatible with bacterial isolates and one with fungal isolate and both actinomycetes were found compatible with each other. Based on the compatibility among the various isolates, five fungi, two bacteria, and two actinomycetes were selected for the consortium.

Studies were further carried out for the identification of promising endophytic microorganisms. The fungal endophytes were identified based on cultural and morphological characters. Accordingly, VRF-1 was identified as *Penicillium melini* and others as *T. harzianum-1* (VSF-3), *T. viride-1* (CSF-1), *T. viride-2* (MyRF-1) and *T. harzianum-2* (ASF-3). Kurian (2011) reported endophytic *Penicillium* sp. from cacao. The endophytic *Trichoderma* has not been reported so far from tomato, however, there are reports with other crops like black pepper (Mathew, 2007), cacao (Bailey *et al.*, 2008), maize (Sobowale *et al.*, 2007), amaranth (Uppala, 2011) and

mangrove plant (Suciatmih and Rahmansyah, 2013). The bacterial isolates were identified as *Bacillus subtilis* (VSB-1) and *Serratia marcesans* (TRB-1). These bacterial endophytes were already reported as endophytes from various crops and also proved to be efficient antagonists. Many species of endophytic *Bacillus* were isolated from sweet corn, cotton, red clover and chestnut (McInroy and Kloepper, 1996). It is further supported by Mathew (2006b) who reported antagonistic endophyte *B. megaterium* from black pepper against *R. solanacearum*. Nawangsih *et al.* (2011) reported *B. amyloliquefacians* as an antagonistic endophyte in tomato against the bacterial wilt pathogen. An endophytic *Bacillus* sp. was also isolated from tomato which showed antagonistic activity against *Fusarium oxysporum* f. sp *lycopersici*. Similarly, Kurian (2011) also isolated endophytic *B. subtilis* from cacao which is antagonistic against *P. palmivora*. The selected endophytic actinomycetes, ORA-1 was identified as *Streptomyces thermodiastaticus* and VRA-1 as *S. griseous* and this result confirmed the earlier report of Sreeja (2011) who also observed *S. thermodiastaticus* as an efficient endophytic actinomycete of tomato against the bacterial wilt pathogen. Tan *et al.* (2006) also observed that, most of the isolates of endophytic actinomycetes obtained from healthy and wilted tomato plants belonged to *Streptomyces*.

Recently, much importance is given for the combined application of the bioagents, as it provided better management of diseases. Several workers have observed efficient disease control and increased plant growth parameters with rhizospheric consortia. However, the information on the effect of endophytic consortial application is meager and scanty. Hence, due importance has been given for the endophytic microbial consortia. Based on antagonistic potential, mutual compatibility, type and species of endophytic microorganisms, five different microbial consortia were formulated consisted of four to six microorganisms and evaluated against bacterial wilt disease under *in planta* condition. Among the five, the consortium consisted of *T. harzianum-1*, *T. viride* -1, *T. viride* -2, *B. subtilis* and

S. thermodiastaticus was found most effective, as it recorded minimum wilt incidence (26.19 %) and was selected for the pot and field experiments.

In planta experiment carried out to find out the effect of microbial consortium along with individual isolates in suppression of bacterial wilt revealed good results as the consortium treated plants showed lowest wilt incidence of 12.5 per cent at 10 days of inoculation against 22.22 to 44.44 per cent in individual applications in addition to the promotion of plant growth. Among the individual endophytic applications, *T. viride-2* recorded lowest incidence of 22.22 per cent against 87.5 per cent in control. This supports the earlier reports that, combination of bio-control agents with different mechanisms will have an additive effect in enhancing disease control and biometric characters compared to their individual application (Guetsky *et al.*, 2002). Jetiyanon and Kloepper (2002) also proposed consortial application of different biocontrol agents for improved and stable control against a complex disease. The reduction in population of *Meloidogyne incognita* and *R. solanacearum* complex and improvement in plant growth characters were noticed in co-inoculation of *T. harzianum* and *P. fluorescens* as compared to individual application and control.

The next aspect of investigation was the evaluation of microbial consortium against *R. solanacearum* under pot culture condition adopting different methods of application. Even though all the six methods of application were found effective, seed treatment + seedling dip + soil application at 45 days after planting was found to be the effective one with minimum per cent incidence of 11.11 per cent at 21 days after inoculation against 80.55 in control. Seedling dip alone as well as seed treatment + seedling dip were also found on par with the aforesaid method. Similar studies have been carried out in various crops. The present result is in agreement with Manimala (2003) who observed maximum suppression of bacterial wilt in solanaceous vegetables with seed treatment + root dipping + soil application of bioagents. Similarly, Chakravarty and Kalita (2012) also noticed lowest wilt incidence in brinjal with the same method of application of *P. fluorescens*. In addition, Sivakumar *et al.*

(2011) reported effective suppression of bacterial wilt of brinjal with seed treatment + seedling root dip + soil application + foliar spray of *B. megaterium*. Akbar (2002) also noticed that, the seed treatment + soil drenching of *P. aeruginosa* was more effective than single application in reducing the wilt incidence in tomato. Thus the pot culture experiment also showed the efficacy of microbial consortium in the management of bacterial wilt disease. The possible factors attributed for the low wilt incidence in pots may be due to less root injury and low temperature. Moreover, the enhancement of soil temperature with irrigation in pot which is almost similar to that of solarised soil, and is always more than that of the normal soil, (> 10^0C) which may be unfavourable for the multiplication and survival of pathogens that may also reduce the wilt incidence.

Both consortial and individual endophytes were applied to the soil and the endophytes were reisolated from consortium as well as individual applications at 60 days after planting and at the time of harvest which showed the population of all applied endophytes from soil, root and stem in both *in planta* and pot culture experiment which indicated the endophytic nature and the survivability of the tested isolates in the soil. Similarly, Mathew (2007) also reisolated *T. viride* and *T. pseudokoningii* from soil, root and stem of black pepper on application of endophytes to the soil. Sobowale *et al.* (2007) also reisolated endophytic *T. pseudokoningii* and *T. harzianum* from maize, effective against *F. verticilloides* and stated that, reisolation of any of the *Trichoderma* species from different points within maize stem other than point of inoculation would be suggestive of their endophytic ability.

In the study on the effect of microbial consortium on seedling vigor of tomato, the consortium treated ones yielded early and high germination per cent as compared to control. Seedling height and vigour index of treated seeds showed 49.86 percent and 69.21 percent increase over control respectively. The result is in accordance with the findings of Sundaramoorthy and Balabaskar (2012) who reported that, the

treatment with combination of *P. fluorescens* and *B. subtilis* showed significantly higher vigour index of tomato seedlings than the individual treatments and the control. Similarly, Raupach and Kloepper (1998) also observed intensive plant growth promotion in cucumber with seed treatment of a mixture of three different PGPR.

The endophytic isolates were further subjected to various tests for understanding the mechanism of action. Consequently, they were tested for the production of volatile and nonvolatile metabolites, siderophore and salicylic acid production. Production of volatile and nonvolatile metabolites play an important role in the antagonistic activity of bioagents against the pathogens. In the present study, 15.51- cent per cent inhibition of *R. solanacearum* was observed by way of volatile metabolites. All the endophytic isolates produced volatile metabolites of which *B. subtilis* showed cent per cent inhibition. Nejad and Johnson (2000) observed that, most of the endophytic isolates from oil seed rape produced volatile metabolites with fungal inhibitory action. Endophytic bacteria including *Pseudomonas putida* and *B. subtilis* (Kurian, 2011), endophytic *Trichoderma* isolated from lentil (Rinu *et al.*, 2014) as well as endophytic actinomycetes (Sreeja, 2011) were also proved to be volatile metabolite producers, thus supporting our results. Wilkins *et al.* (2000) reported the production of volatile metabolites of 2-propanol, 3-methylfuran, methyl-1-propanol, 1-pentanol, and 2-hexanone by *T. viride*. Rini and Sulochana (2007) also noticed 54 per cent inhibition of *R. solani* and 48 per cent of *F. oxysporum* with the volatile metabolites of *Trichoderma.*

HCN is considered as a possible and perhaps frequent mechanism by which bacteria suppress plant pathogens. However, in the present study none of the endophytic isolates were found to produce HCN. This result was confirmed by the earlier report by Nejad and Johnson (2000) who found that, most of the endophytic isolates from oil seed rape were HCN negative and suggested that, volatiles other than HCN may be involved in the antagonism. Kurian (2011) and Sreeja (2011) also

not observed the cyanogenic nature of the endophytic bacteria or actinomycetes used in their study, thus supporting the present results.

The capacity to produce ammonia is an attribute, which is directly related to antagonistic ability of a biocontrol organism. In the present investigation, ammonia production was observed in all the three types of endophytic organisms and it was more in bacteria as compared to fungi and actinomycetes with maximum in *B. subtilis*. The ammonia production by certain endophytic bacteria have been observed by Balan (2009) and Kurian (2011) against *X. axonopodis* of anthurium and *P. palmivora* of cocoa respectively.

Non-volatile metabolite production of endophytes was studied by adopting both cellophane paper and culture filtrate methods, which showed complete inhibition of *R. solanacearum* indicating that the main mechanism of antagonism is by antibiosis. This is in accordance with the reports of Bacon and Hinton (2002) and Kurian (2011) who reported the production of diffusible inhibitory metabolites in the medium by endophytic bacteria including *B. subtilis*. *B. subtilis* produces iturin, bacilycin and fengacin antibiotics which can inhibit various fungal and bacterial pathogens. Manimala (2003) also reported that, culture filtrates of *B. subtilis* and *T. viride* had good inhibitory effect on *R. solanacearum* of tomato. Rini and Sulochana (2006) observed the suppression of different pathogens infecting tomato by the nonvolatile metabolites produced by *Trichoderma* spp. Sreeja (2011) studied the production of non-volatiles by endophytic actinomycetes. Thus these reports also confirmed the present findings.

The production of siderophores by microorganisms is beneficial to plants as it can inhibit the growth of plant pathogens. Ability to selectively chelate iron for own purpose thus making it unavailable for others is a well known mechanism by which antagonistic bacteria limit the growth of pathogenic microbes. In the present study, none of the endophytic isolates showed siderophore production. This is in

contradictory to the earlier findings of Balan (2009), Kurian (2011) and Sreeja (2011) who observed siderophore production by bacteria and actinomycete endophytes. It is well documented that, fluorescent pseudomonads produce maximum amount of siderophores as compared to other bacterial species. The bacterial endophyte, *B. subtilis* obtained in the present study did not produce siderophore. Sgroy *et al.* (2009) also reported that, endophytic *B. subtilis* isolate from halophyte *Prosopis strombulifera* did not produce siderophores, thus supported the present result. Forchetti *et al.* (2010) also not observed siderophore production by bacterial endophytes from sunflower.

An attempt was made to detect the production of salicylic acid by the endophytic isolates, as it plays a significant role in imparting resistance and thereby suppressing the diseases. Quantitative estimation of salicylic acid from cell free culture filtrates of endophytes revealed the production of SA in varying quantities with maximum content in *B. subtilis*. Bacterial isolates exhibited more amount of salicylic acid production as compared to fungi and actinomycetes. Several workers reported the role of salicylic acid in induction of systemic resistance in various crops by application of PGPR. (De Meyer and Hofte, 1997; De Meyer *et al*, 1999; Vijayaraghavan, 2007). Endophytic bacteria from tomato belonging to *Bacillus*, *Pseudomonas*, *Klebsiella* and *Citrobacter* were found to produce salicylic acid *in vitro* (Nandhini *et al.*, 2012).

Recalling back the results on the inhibitory effect of *B. subtilis in vitro* and *in planta* experiments, it is seen that *B. subtilis* had the ability to produce high amount of volatile and nonvolatile metabolites and salicylic acid which may be the mechanisms involved in the antagonism.

Role of IAA in plant growth promotion is a known phenomenon which can lead to enhancement of resistance to various pathogens. Production of phytohormones as one of the mechanisms involved in plant growth promotion by

endophytes has been suggested by Sturz *et al.* (2000) and Sessitech *et al.* (2002). Hence the potential endophytic isolates were tested for their ability to produce IAA. All the five isolates used in the present study were found to produce varying levels of IAA ranged from 10.67 to 192.17 µg/ml with maximum by *T. harzianum*. The fungal endophytes produced higher amount of IAA compared to bacterial endophytes. This is in accordance with findings of Waqas *et al.* (2012) who observed that, endophytic fungal strains obtained from cucumber varied greatly in their inherent ability to produce IAA. PGPR isolates including *Bacillus* spp and *Pseudomonas* spp were also reported to produce IAA in varying levels (Vijayaraghavan, 2007). Similarly, Kurian (2011) and Sreeja (2011) also noticed IAA production by endophytic bacteria, fungi and actinomycetes.

Next point of investigation was to find out the effect of secondary metabolites on the pathogen. Endophytes constitute a valuable source of secondary metabolites (Miller, 1995). Secondary metabolites such as alkaloids, terpenoids, sterols and phenolic compounds are the constituents which play a major role in plant defense mechanisms (Nicholson and Hammerschmidt, 1992; Kuc, 1995). Therefore, an experiment was carried out to study the effect of secondary metabolites of endophytes against the pathogen both *in vitro* and *in vivo* conditions. The selected endophytes tested *in vitro* were found positive for the production of inhibitory substances recording cent percent inhibition of the pathogen. Hence these endophytes were tested *in vivo*, using culture filtrate along with culture suspension for comparison. Among the different treatments, dipping the seedlings either in culture filtrate or suspension prior to bacterial ooze treatment was more effective as it recorded lowest wilt incidence compared to dipping after bacterial inoculation. It may indicate the preventive or protective action by the way of antibiosis and defense mechanism of the endophytes. Among these two, dipping first in culture filtrate was found to be the best treatment with minimum incidence noted in filtrate of *T. viride*-2 (19.84 %), which may be due to quick and direct action of secondary metabolites in the culture filtrate

on the pathogen. It is also noted that, seedlings treated first in ooze and later in suspension/culture filtrate also showed lesser disease incidence as compared to control which indicate curative or suppressive action of filtrate/suspension after entry of the pathogen. It is also noted that, culture filtrate of *T. viride*-2 (a root endophyte from Mannuthy) and suspension of *B. subtilis* (a stem endophyte from Vellanikkara) recorded minimum incidence of 19.84 and 26.19 per cent respectively which may be due to the high amount of secondary metabolites in the fungal filtrate and the faster multiplication of bacterial cells in case of *B. subtilis*. Thus this study indicated the role of secondary metabolites of the endophytes in enhancing the defense mechanism in tomato. A search through the literature did not give relevant information about the aforesaid aspects. Mehra (2008) reported that, tomato seedlings dipped in culture filtrate of *T. harzianum* showed maximum control of Fusarium wilt after inoculation. Endophytic bacterial strains also have been reported to produce many kinds of metabolites which can play an important role in ISR against many plant diseases (Mavrodi *et al.*, 2001). The culture filtrate effect of endophytic *Bacillus* sp. was studied against different plant pathogens of rice and observed that, *Bacillus* strains produced several cyclic peptides, amino-polyols and amino-glycosides, which are having significant effect on ISR development (Yu *et al.*, 2002). The exact mechanism by which endophytic microorganisms induce protection in the host plants remains unclear, although production of secondary metabolites has been suggested as a possible mechanism. An intensive research is necessary to find out the facts and factors involved in the exact mechanism, which was beyond the scope of the present investigation.

Considering the overall performance of five endophytes, *B. subtilis*, the stem endophyte from Vellanikkara, was found to be the most efficient endophyte with respect to inhibitory effect in *in vitro* and *in vivo*, mode of action by volatile metabolites and secondary metabolites and salicylic acid production. It is followed by *T. viride*-2, the root endophyte from Mannuthy was also equally effective as

B. subtilis except in salicylic acid production. *T. viride-1*, the stem endophyte from Cherumkuzhy, was efficient with respect to salicylic acid. *T. harzianum-1*, the stem endophyte from Vellanikkara, showed high IAA production and actinomycete was found effective only in the production of nonvolatile metabolite. The present study also revealed the possibility of exploiting endophytic *Trichoderma* spp. for the management of diseases, as endophytes are well protected and show better ability to survive.

All the plants possess active defense mechanisms against the attack of pathogens. These active defense mechanisms will be failed when the plant is infected by a virulent pathogen because the pathogen avoids triggering or suppresses resistance reaction or evades the effects of active defenses. If the defense mechanisms are triggered by a stimulus prior to infection by a plant pathogen, disease can be reduced (Van Loon *et al.*, 1998). Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated (Kuc, 1982). Induced systemic resistance (ISR) is suggested as the major mechanism involved in the biocontrol by endophytes and indirect way of suppression of diseases (Adhikari *et al.*, 2001). The endophyte mediated resistance was found to be effective over time indicating persistence, and is hypothesized to be a form of induced resistance (Ganley *et al.*, 2008). A search through the relevant literature did not give any information regarding the consortial effect of microbes on ISR mechanism. Since microbial consortium contains microbes having different activities, the application of consortium can enhance the defense mechanism much better than the individual isolates. Keeping all these in mind, an experiment was taken up to study the defense mechanism induced by endophytic consortium and to assay the defense related compounds produced after inoculation of the pathogen.

Phenolic compounds are known to play a major role in the defense mechanism of plants by enhancing mechanical strength of cell wall and also by inhibiting the invading pathogens thereby conferring resistance to the plants either

directly or indirectly through activation of post infection responses in hosts. In the present investigation, total phenol content was found more in stem than in roots of tomato plants. The lower level of phenolics in the roots might be due to the increased rate of oxidation of phenolics to more toxic compounds like quinones by the oxidative enzymes like polyphenol oxidase and peroxidase (Mahadevan, 1970). Since the bacteria colonize and multiply in the vascular tissues, the higher phenolic content in the stem can provide resistance to the invasion of pathogen in plants. In view of this, the effect of phenols in the plants was estimated before and after challenge inoculation.

Plants treated with endophytic consortium showed higher accumulation of total phenols in both roots and stem compared to control, prior to inoculation of the pathogen. An increasing trend was observed upto five days after challenge inoculation and thereafter declined. Higher accumulation of phenolics by prior application of *P. fluorescens* challenged with the pathogen has been reported in various crops (Meena *et al.*, 2000; Vivekananthan *et al.*, 2004). Malinowski *et al.* (1998) reported 20 per cent more total phenolic concentration in endophyte infected plants than control plants. Rajendran *et al.* (2006) also reported enhanced mechanical strength of host cell walls and inhibition of invading *Xanthomanas axonopodis* pv. *malvacearum* in cotton by endophyte treatment. The treatment with microbial consortia resulted in higher accumulation of phenols in pea leaves compared with untreated plants challenged or unchallenged with *Sclerotinia sclerotiorum* (Jain *et al.*, 2012).

Higher OD phenol content was noticed in tomato plants treated with consortium compared to control. The OD phenolic content also showed increasing trend upto five days after inoculation which later declined on 10^{th} day of inoculation. This is in accordance with Tomiyama (1963) who reported that, mono and dihydric phenols increased in host tissues invaded by parasites as a part of resistance mechanism. The high toxicity of OD phenols and its role in resistance was also

observed by Mahadevan (1966). He reported that, orthodihydroxy phenolic compounds such as caffeic acid, and chlorogenic acid, and orthoquinones and tannins were shown to strongly inhibit the activities of extracellular enzymes produced by microorganisms.

Activation of defense related enzymes is one of the major physiological changes that occur in plants in response to infection by pathogen or stimulation by biocontrol organism. Though these enzymes are not directly toxic to the pathogen, enhanced activity of these enzymes is directly correlated with defense reaction in plants. Hence the next point of consideration was the assay of oxidative enzymes *viz.* peroxidase, polyphenol oxidase and phenylalanine ammonia lyase using spectral analysis to determine the effect of endophytic microorganisms in inducing systemic resistance.

Peroxidase is a phenol oxidizing enzyme which oxidizes phenols to form quinones and also generate hydrogen peroxide. It is a key enzyme in the biosynthesis of lignin (Bruce and West, 1989; Brisson *et al.*, 1994). The present study showed higher activity of peroxidase enzyme in both root and stem samples of consortium treated plants at five days after inoculation especially in consortium treated plants with pathogen. In challenge inoculated treatments, the enzyme activity was found to decrease at 10^{th} day after inoculation. This is in confirmation with the findings of Kurian (2011) who also reported increased PO activity in cocoa upto five days after inoculation and later decreasing trend in all treatments with endophytes. Uppala (2007) also reported enhanced activity of peroxidase in amaranth by endophyte application. Various studies reported that, PGPR induced peroxidase in response to pathogen attack (Vijayaraghavan, 2007; Anita and Samiyappan, 2012).

Similar to PO activity, PPO activity also increased in tomato plants treated with consortium but maximum in consortium treated plants with pathogen at five days after inoculation. Though this enzyme activity was found declined at 10^{th} day

after inoculation, the activity was found at higher level compared to control. There are earlier reports of enhanced levels of PPO by endophyte treatment in cotton against *X. malvacearum* (Rajendran *et al.*, 2006); in black pepper against *P. capsici* (Barka *et al.*, 2002) with reduction in disease. Enhanced activity of PO and PPO were observed in tomato roots treated with *P. fluorescens* and *Fol* (Ramamoorthy *et al.*, 2001). Chen *et al.* (2000) also reported similar enhanced activity of PPO in cucumber root treated with various rhizobacteria and the pathogen, *P. aphanidermatum*.

Phenylalanine Ammonia Lyase (PAL) is another enzyme involved in lignification and induced systemic resistance. Daayf *et al.* (1997) reported that, PAL is a key enzyme in the production of phenols and phytoalexins. In this study, steady increase in PAL activity was observed in plants treated with consortium and pathogen and the enzyme activity was found declined after five days of inoculation but with higher activity compared to control. A similar trend of enzyme activity was observed in both stem and root. The treatment with medium alone did not show any noticeable variation in the enzyme activity during the periods of observation. No literature was available regarding the role of medium in inducing resistance. Chen *et al.* (2000) noticed high levels of PAL in cucumber roots treated with *P. corrugata* and later decreased after inoculation of *P. aphanidermatum*. Uppala (2007) observed increased PAL activity in amaranth with treatments of endophytes. Induction of phenols is also linked with induced PAL activity, which catalyses the first step in synthesis of phenols. Increased PAL activity and phenol accumulation in the present study may, thus be correlated with enhanced defense response by the microbial consortium.

PR proteins are host-coded proteins induced by different types of pathogens and abiotic stresses, and their synthesis and accumulation have been reported to play an important role in plant defense. Several monocot and dicot plants have been found to produce PRs through a ubiquitous reaction during pathogen attack. They have been associated with systemic acquired resistance and incipient anti-pathogen effects. Collective function of several PR proteins may be effective in inhibiting pathogen

growth, multiplication and spread of pathogen and be responsible for the state of induced resistance (Van Loon, 1997). The results of the present study indicated that, endophytic consortium treated plants showed higher activities of β-1,3-glucanase upto 10 days after inoculation but in plants inoculated with consortium and pathogen, the activity reduced after five days of inoculation. This is in line with the result of Kurian (2011) who also observed enhanced activity of β-1,3-glucanase induced by endophytes in cocoa on challenge inoculation with *P. palmivora*. Increased β-1,3-glucanase activity on application of endophyte, *B. subtilis* has also been confirmed by Wilhelm *et al.* (1998) in chestnut against chestnut blight and Jayaraj *et al.* (2004) in rice against sheath blight.

The present investigation also showed increased activity of chitinase in consortium treated plants. The plant chitinases can utilize bacterial peptidoglycan as a substrate and can hydrolyze β-1,4-linkages between N-acetyl muramic acid and G1cN1Ac residues in peptidoglycan (Majeau *et al.*, 1990). Therefore increased activity of chitinase can provide resistance to bacterial wilt pathogen. The activity was found decreasing in all treatments after five days of inoculation and this clearly depicts the fact that, the chitinase activity is pronounced only for a shorter period and later decline. Similar results of increase in PR proteins after application with biocontrol agents have been reported by several workers in different crops (Kuc, 1995; De Meyer *et al.*, 1998, Meena *et al.*, 2000). Thus, the present findings on the effect of secondary metabolite and ISR studies supported the report of Mavrodi *et al.* (2001) who stated that, the endophytic bacterial strains produced many kinds of metabolites which could play an important role in ISR against many plant diseases. Therefore, the study reveals the potential of endophytic microbial consortium in enhancing the plant's own defense mechanism against the bacterial wilt pathogen.

An optimal recommendation of a biocontrol agent for the management of a disease could be brought forth only when applying under field conditions. The natural environment will be definitely a limiting factor. A biocontrol agent which is more

effective even in such adverse conditions may be termed stable. With this view, an investigation was conducted to study the efficacy of endophytic consortium under field condition with four different treatments and three varieties, for the better understanding of the establishment of endophytes under natural condition and also its impact in enhancing resistance in these varieties. The treatments consisted of endophytic consortium of *T. harzianum*-1, *T. viride*-1, *T. viride*-2, *B. subtilis*, and *S. thermodiastaticus*, rhizosphere consortium of *T. harzianum* and *P. fluorescens*, KAU reference culture, *P. fluorescens*, and copper hydroxide (2g/l) as a comparison check along with absolute control. The biocontrol treatments were applied as seed treatment + seedling dip + soil application 45 DAP and copper hydroxide as soil drenching at the time of planting and 45 DAP in three different varieties, highly susceptible PKM-1, susceptible F1 hybrid COTH-3 and resistant variety, Anagha. Among the different treatments, soil drenching with copper hydroxide (2g/l) was found most effective in reducing the wilt incidence in all the three varieties. It is well known about the efficacy of copper hydroxide in reducing the bacterial wilt incidence in solanaceous vegetables. Chemical control is always better than any other management practices like cultural and biological methods. So the effect of copper hydroxide in controlling the disease has not been given much priority in the present study since our main aim was to study the role of microbial consortium in reducing the wilt incidence. Considering the biocontrol aspects of the study, the endophytic consortium was found most effective at 60 days after planting as compared to rhizosphere consortium and *P. fluorescens* in varieties PKM-1 and COTH-3. Since the endophytic consortium consisted of five organisms and rhizosphere of only two, the multiple modes of action and synergistic effect of more number of organisms in endophytic consortium can also be one of the reasons for such reduction as compared to rhizospheric consortium and *P. fluorescens*. It is also a fact that, consortial application is always better than individual application. Moreover, the endophytic organisms reside within the plants that, competition with other microorganisms is

less, and the environment is congenial for the multiplication of endophytes within the plant. The phenomenon of ISR is more pronounced in the case of endophytic microbes compared to other rhizosphere organisms.

From the field experiment conducted, it was found that, all the treatments were superior to control with respect to disease incidence and biometric characters in all the three varieties. In highly susceptible variety PKM-1, plants treated with endophytic consortium showed 58.33 per cent incidence against 98.61 in control recording 40.85 per cent reduction at 60 days after planting. Even though COTH-3 is a susceptible hybrid variety, in the present study, wilt incidence could be reduced to certain extent by the application of endophytic consortium and this treatment showed minimum disease incidence of 36.11 against 68.06 per cent in control recording 46.94 per cent reduction at 60 DAP. Soil application of the microbial consortium again at 45 days after planting can also be one of the attributing factors for the disease reduction by improving the defense mechanism in the later stage. On recalling the results of induced systemic resistance, it is evident that, the activity of defense related compounds in plants was enhanced by the application of the endophytic consortium, thus improved the level of defense mechanism thereby reduced the disease progression.

F_1 hybrid varieties are very much preferred for the polyhouse cultivation in Kerala due to their high yielding performance. But the limitation for the cultivation of F_1 hybrid is its susceptibility to bacterial wilt disease which can be overcome with the present findings. In highly resistant variety, Anagha, the wilt incidence was very less in all treatments. The effect of endophytic consortium in a resistant variety like Anagha is not so pronounced as in susceptible variety except in the improvement of the biometric characters. Mukthi, a tomato variety which was considered as resistant variety in Kerala has now become moderately resistant due to fluctuating nature and variability of the pathogen. So, an attempt was made to improve its resistance by activating the defense mechanism through endophytic consortium. It was interesting

to note that, the microbial consortium reduced the disease incidence by 52.81 per cent. In the present investigation, a combination of host resistance and application of microbial consortium revealed better management of bacterial wilt disease thus supporting the findings of Manimala (2003). It is also in agreement with the earlier reports of Guetsky *et al.* (2002) and Jetiyanon and Kloepper (2002) that, consortial application of different bioagents is required for improved and stable control against a complex of disease and noticed reduction in population of pathogen and improvement in plant growth characters with co-inoculation of bioagents as compared to individual application and control. Consortial effect of rhizospheric antagonists against Phytophthora rot of black pepper and vanilla and bacterial wilt disease of chilli and ginger under field condition have also been studied by Mathew (2008 & 2009).

In reviewing the effect of antagonists *in toto*, showed the drop in inhibition on pathogen when the study is gradually shifted from *in vitro* set up to pot culture and field conditions. Positively, the change from micro environment to macro environment is evident. Root injuries that occur during the cultural practices facilitate easy entry of the pathogen, spread of the bacterium through irrigation water, high soil temperature, and high inoculum level in the soil are some of the factors that favour high wilt incidence in the field. Biological control using rhizospheric microorganisms is mainly affected by the competition with other soil pathogens which to a certain extent could be overcome by endophytic microorganisms.

The effectiveness of a disease management strategy will be complete, when it coincides with the increase in crop yield. As a matter of fact, endophytic consortium contributes maximum towards the enhancement of crop yield in susceptible, resistant and moderately resistant varieties. In the present study, plot treated with endophytic consortium recorded maximum yield of 2.67 kg, 8.62 kg and 6.38 kg/ 8.64 m^2 against 0.25 kg, 3.25 kg and 2.08 kg/8.64 m^2 in control in varieties, PKM-1, COTH-3 and Mukthi respectively. Therefore, considering the overall performances of various

treatments, endophytic consortium was found effective in suppressing wilt disease and in promoting plant growth characters.

Reading back the results obtained with respect to the change in biochemical activities in plants and the effect of secondary metabolites of the endophytes on *R. solanacearum*, it is evident that, the application of microbial consortium can enhance the defense mechanism in plants by the way of increased activities of phenols, oxidative enzymes and PR proteins which are the vital factors for imparting resistance against plant pathogens. Search on literature revealed few studies in India regarding the effect of endophytic microbial consortium against plant pathogen, that also with the combination of two bacteria. It is worthwhile to mention that, perhaps this may be the first study on endophytic microbial consortium with a combination of fungi, bacteria and actinomycetes. Various workers have studied the effect of endophytes on *R. solanacearum* but the attempt to exploit the role of endophytic microbial consortium against *R. solanacearum* is for the first time in the world.

It may be concluded that, the present study has enriched our knowledge on endophytes and thrown light on the role of microbial communities in the management of a dreadful soil borne disease.

Summary

6. SUMMARY

Bacterial wilt caused by *Ralstonia solanacearum* is one of the major constraints for the cultivation of tomato in Kerala and cent per cent crop loss has been estimated in susceptible varieties. *R. solanacearum* is a genetically diverse soil borne pathogen with wide host range and difficult to control once established in the field. The focus on the management of plant diseases has been shifted from chemical pesticides to more ecofriendly biopesticides to reduce health hazards and environmental pollution. Biological control of plant disease is becoming increasingly important as a potential mode to manage the diseases. Among the biological control strategies, utilization of potential antagonistic endophytes is considered as a novel approach for efficient disease management due to their intimate systemic association with the plants. Recently, a greater thrust is being given for the development of bioconsortium, as it provides better management of diseases by the way of synergistic effect and multiple modes of action. In this view, an investigation was carried out to study the effect of endophytic consortium on the bacterial wilt pathogen, *R. solanacearum*. The salient findings of the present study are summarized below.

- Healthy tomato plants were collected from 16 different locations of Kerala for the isolation of endophytes from root and stem. Quantitative estimation of the endophytic microorganisms revealed that, the microbial population was more in root compared to stem and the bacterial population was higher than fungi and actinomycetes in both root and stem.

- Maximum population of bacteria and fungi were recorded in root samples of Alappuzha and Kumarakom respectively whereas, actinomycetes population was highest in the root samples of Panniyur, Ozhalapathy and Amburi.

- A total of 154 predominant colonies of endophytes, of which 79 bacteria, 68 fungi, and seven actinomycetes, were isolated from tomato plants from different locations. Among these, 12 bacteria, 16 fungi, and four

actinomycetes were found highly antagonistic to *R. solanacearum* under *in vitro* condition, of which, five bacteria, eight fungi, and two actinomycetes were found promising in *in planta* experiment.

- Five fungal isolates, *viz.* MyRF-1, CSF-1, ASF-3, VRF-1 and VSF-3, two bacteria, VSB-1 and TRB-1, and two actinomycetes, VRA-1 and ORA-1 were found mutually compatible to each other and were selected for the development of different consortia.

- Endophytes selected for the consortia were identified as *Trichoderma viride*-1 (MyRF-1), *T. viride-2* (CSF-1), *T. harzianum*-1 (VSF-3), *T. harzianum*-2 (ASF-3), and *Penicillium melini* (VRF-1), bacteria as *Bacillus subtilis* (VSB-1) and *Serratia marcesans* (TRB-1), and actinomycetes as *Streptomyces thermodiastaticus* (ORA-1) and *S. griseous* (VRA-1).

- Among the five microbial consortia tested, the consortium consisted of *T. viride*-1 (MyRF-1), *T. viride-2* (CSF-1), *T. harzianum*-1 (VSF-3), *B. subtilis* (VSB-1), and *S. thermodiastaticus* (ORA-1) was the most effective.

- In a comparison study, the consortium of endophytes was found more effective than individuals in reducing bacterial wilt incidence.

- In pot culture evaluation, the endophytic consortium applied as seed treatment + seedling dip + soil application at 45 days after planting showed minimum wilt incidence.

- The endophytic consortium treated seedlings showed 69.21 per cent increase in vigour index as compared to control.

- All endophytic isolates showed positive reaction towards ammonia production but negative for HCN and siderophore production. Volatile metabolites of the endophytes showed 15.51 to cent per cent inhibition of the pathogen with

maximum by *B. subtilis*. Nonvolatile metabolites of the endophytes showed complete inhibition of the pathogen.

- All endophytes showed IAA production which ranged from 34 to 192.17 µg ml^{-1} with maximum in *T. harzianum*-1. Salicylic acid production varied from 3.42 to 23.48 µg ml^{-1} with maximum by *B. subtilis*.

- Study on the effect of secondary metabolites on wilt incidence showed that, seedlings dipped in culture filtrate of endophytes for 2 h and later dipped in bacterial ooze for 30 min before planting showed, lowest per cent wilt incidence ranging from 19.84 to 38.89 with minimum for *T. viride*-2 against cent per cent in control.

- Total and OD phenolic contents were more in stem as compared to roots. Whereas, the activity of oxidative enzymes and PR proteins were higher in roots than stem. Total and OD phenols, activities of peroxidase, polyphenol oxidase, and phenyl alanine ammonia lyase and β-1,3-glucanase and chitinase were found higher in consortium treated plants with maximum in plants inoculated with both consortium and pathogen.

- Application of endophytic consortium recorded 40.85 per cent disease reduction in a highly susceptible variety PKM-1, and 46.94 per cent reduction in susceptible hybrid variety, COTH-3 whereas in highly resistant variety, Anagha, wilt incidence was comparatively less that, any noticeable difference could be observed among the treatments. In moderately resistant variety, Mukthi, application of the endophytic consortium was very effective showing 52.81 per cent efficiency over control under field condition.

Thus the present study has revealed the fact that, the application of endophytic microbial communities will enhance the resistance mechanism in susceptible and moderately resistant varieties of tomato against the bacterial

wilt pathogen. Moreover, this study also indicates the suitability of high yielding and susceptible tomato hybrids for the protected cultivation in Kerala with the application of microbial consortium. The findings of the present study may open up a new approach for the management of bacterial wilt by endophytic microbial communities.

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Appendices

APPENDIX I

Composition of different media

1. Nutrient Agar medium (NA) (Lapage *et al.*, 1970)

Peptone	:	5.0 g
Beef extract	:	1.0 g
Sodium Chloride	:	5.0 g
Agar	:	20.0 g
Distilled water	:	1000 ml
pH	:	6.5 - 7

2. King's B medium (King *et al.*, 1954)

Peptone	:	20.0 g
Glycerol	:	10.0 ml
K_2HPO_4	:	10.0 g
$MgSO_4.7H_2O$	:	1.5 g
Agar	:	20.0 g
Distilled water	:	1000 ml
pH	:	7.2 – 7.4

3. Martin's Rose Bengal Agar medium (Martin, 1950)

Dextrose	:	10.0 g
Peptone	:	5.0 g
KH_2PO_4	:	1.0 g
$MgSO_4$	:	0.5 g
Agar	:	20.0 g
Rose Bengal	:	0.03 g
Streptomycin	:	30.0 mg (added aseptically)
Distilled water	:	1000 ml

4. Trichoderma Selective Medium (TSM) (Elad *et al.*, 1981)

Glucose	:	3.0 g
NH_4NO_3	:	1.0 g
KCl	:	0.15 g
K_2HPO_4	:	1.0 g
$MgSO_4.\ 7H_2O$	:	0.5 g
Agar	:	15 g
Distilled water	:	1000 ml

5. Kenknight's Agar medium (Subba Rao, 1977)

Dextrose	:	1.0 g
KH_2PO_4	:	0.1 g
$NaNO_3$	:	0.1 g
KCl	:	0.1g
$MgSO_4$	:	0.1g
Agar	:	20.0 g
Distilled water	:	1000 ml
pH	:	7.0

6. Potato Dextrose Agar medium (PDA) (Beever and Bollard, 1970)

Potato	:	200.0 g
Dextrose	:	20.0 g
Agar	:	20.0 g
Distilled water	:	1000 ml

7. Triphenyl Tetrazolium Chloride (TZC) medium (Kelman, 1954)

Peptone	:	10 g
Casein hydrolysate	:	1 g
Glucose	:	5 g
Agar	:	17 g
Distilled water	:	1000 ml
Triphenyl tetrazolium chloride	:	0.005%
pH	:	6.8

8. Peptone water (MacFaddin, 1985)

Peptone	:	10 g
NaCl	:	15 g
Distilled water	:	1000 ml

9. Sodium succinate broth (Meyer and Abdallah, 1978)

KH_2PO_4	:	0.33 g
$MgSO_4.7H_2O$	:	0.33 g
NaCl	:	0.33 g
NH_4Cl	:	0.5 g
$CaCl_2.2H_2O$	:	0.05 g
Sodium succinate	:	1.0 g
Yeast extract	:	0.02 g
Distilled water	:	1000 ml
pH	:	6.8-7.2

10. Luria Bertani (LB) broth (Sambrook and Russell, 2001)

Tryptone	:	10.0 g
Yeast extract	:	5.0 g
NaCl	:	10.0 g
Distilled water	:	1000 ml
pH (at 25°C)	:	7.5±0.2

11. Czapek-Dox broth (Czapek, 1902)

Sucrose	:	30.0 g
Sodium nitrate	:	3.0 g
Magnesium sulfate	:	0.5 g
Potassium chloride	:	0.5 g
Iron sulfate	:	0.01 g
Di-potassium hydrogen phosphate:		1.0
Distilled water	:	1000 ml

12. Yeast extract- Malt extract Agar Medium (YM) (Wickerham, 1951)

Yeast extract	:	4.0 g
Malt extract	:	10 g
Glucose	:	4.0 g
Agar	:	20.0 g
Distilled water	:	1000 ml

13. Casaminoacid broth (Mueller and Johnson, 1941)

Glucose	:	15 g
Casamino acid	:	1.5 g
Yeast extract	:	1 g
KH_2PO_4	:	1.5 g
Distilled water	:	1000 ml

APPENDIX II

COMPOSITION OF BUFFERS USED IN BIOCHEMICAL ANALYSIS

1. **Sodium borate buffer, pH 7.0**

   500 mM NaOH

   120 g H_3BO_3 (boric acid powder) - Added to 500 mM NaOH

   Bring to 1 L with distilled water.

2. **Sodium phosphate buffer, pH 6.5**

   Monobasic: Dissolve 2.78gm of sodium dihydrogen phosphate in 100ml of distilled water.

   Dibasic sodium phosphate (0.2M): Dissolve 5.3gm of disodium hydrogen phosphate or 7.17 gm sodium hydrogen phosphate in 100ml distilled water.

   39 ml of dihydrogen sodium phosphate is mixed with 61 ml of disodium hydrogen phosphate This made up to 200ml with distilled water .This gives phosphate (PO4)2 buffer of 0.2M.

3. **Sodium acetate buffer, pH 4**

   Acetic Acid 0.2M: 1.5 ml of glacial acetic acid is made upto 100ml with distilled water.

   Sodium Acetate Solution: 0.64 gm of sodium acetate or 2.72gm of sodium acetate trihydrate is dissolved in 100ml Distilled water.

   Pipette out exactly 36.2ml of sodium acetate solution into 100ml of standard flask and add 14.8ml of glacial acetic acid, make the volume 100ml using distilled water using distilled water. This gives 0.2 M of acetic acid and sodium acetate buffer. The pH is measured with pH meter.

4. **Sodium citrate buffer, pH 5**

Citric acid: Dissolve 2.101 gm of citric acid in 100ml distilled water.

Sodium citrate solution 0.1 M: Dissolve 2.941gm of sodium citrate in 100ml distilled water.

46.5ml of citric acid with 3.5ml of sodium citrate solution and upto 100ml with distilled water. It corresponds to 0.1 M citrate buffer and standardised with pH meter and measures the pH of the prepared solution.

5. **Potassium phosphate buffer, pH 7.1**

Dipotassium hydrogen phosphate

Potassium dihydrogen phosphate

174.18 g/mol dipotassium hydrogen phosphate and 136.09 g/mol potassium dihydrogen phosphate was taken and made up to 200ml using distilled water. This gives the potassium buffer.

Standardised pH meter with standard buffer. Washed electrode with distilled water and introduced it into potassium buffer prepared.

ENHANCEMENT OF RESISTANCE TO BACTERIAL WILT IN TOMATO BY ENDOPHYTIC MICROBIAL COMMUNITIES

By

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ABSTRACT OF THE THESIS

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ABSTRACT

The study on "Enhancement of resistance to bacterial wilt in tomato by endophytic microbial communities" was conducted in the Department of Plant Pathology, Department of Agricultural Microbiology, and Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2011-2014.

The endophytes were isolated from root and stem of healthy tomato plants from 16 locations of north, central and south Kerala. Endophytic microbial population varied with the plant samples and the population was more in root than stem. Bacterial population was higher than fungi and actinomycetes in root and stem. Among 154 endophytes isolated, 12 out of 79 bacteria, 16 out of 68 fungi, and four out of seven actinomycetes were antagonistic to *R. solanacearum* in *in vitro*. Among them, five bacteria, eight fungi, and two actinomycetes were promising *in planta*. Mutually compatible endophytic isolates were selected for the development of consortia and these were identified based on cultural, morphological and molecular characters. Of the five consortia tested, the one consisted of *Trichoderma viride-1*, *T. viride-2*, *T. harzianum-1*, *Bacillus subtilis*, and *Streptomyces thermodiastaticus* showed effect in reducing wilt incidence.

Comparative study of the microbial consortium with individual endophytes showed the higher efficacy of consortium in reducing the wilt incidence. The endophytes were reisolated from soil, root, and stem of tomato plants. In pot culture experiment, the consortium applied as seed treatment + seedling dip + soil application at 45 DAP showed the minimum wilt incidence. Studies on the mechanism of antagonism of endophytic isolates showed, positive reaction towards ammonia and negative for HCN and siderophore production. The volatile and nonvolatile metabolites of the endophytes inhibited the pathogen. The endophytes showed varying levels of IAA and salicylic acid production with the maximum in

T. harzianum-1 and *B. subtilis* respectively.

Study on the effect of secondary metabolites of endophytes on the disease indicated that, seedlings dipped first in culture filtrate for 2 h and later dipped in bacterial inoculum for 30 min before planting showed the lowest per cent wilt incidence with the minimum for *T. viride-2.* Induction of systemic resistance was studied by assay of defense related compounds such as phenols, oxidative enzymes and PR proteins. The plants treated with microbial consortium showed higher activity of the defense related compounds with the maximum in plants inoculated with both consortium and pathogen.

Field evaluation of endophytic consortium against bacterial wilt showed reduction in wilt incidence of 40.85 per cent in highly susceptible variety, PKM-1, 46.94 per cent in susceptible F1 hybrid, COTH-3, and 52.81 per cent in moderately resistant variety, Mukthi.

Thus, the study revealed that, the application of endophytic consortium can enhance the resistance mechanism in tomato against bacterial wilt pathogen, *R. solanacearum.*