BIOEFFICACY OF ENDOPHYTIC ACTINOMYCETES ON PLANT GROWTH PROMOTION AND MANAGEMENT OF BACTERIAL WILT IN TOMATO

By

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(2009-11-124)

THESIS

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DECLARATION

I hereby declare that this thesis entitled **"Bioeffficacy of endophytic actinomycetes on plant growth promotion and management of bacterial wilt in tomato"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis, entitled **"Bioeffficacy of endophytic actinomycetes on plant growth promotion and management of bacterial wilt in tomato"** is a record of research work done independently by **Sreeja S.J. (2009-11-124)** 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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Sreeja S.J.


Dedicated
To
My Family

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Introduction

1. INTRODUCTION

Tomato (*Solanum lycopersicum* Mill.) is one of the most popular vegetables in India. It is cooked as a vegetable or taken as raw or used for salad, soup, pickles, sauce, ketchups. The crop occupies about 7.5% of the total vegetable area in India and its share in the total production is about 8.5% (Indian Horticulture Database, 2005). It came from tropical America and spread to the rest of the world in the 16^{th} century. It was perhaps introduced into India by the Portuguese. It is a major source of vitamins and minerals. Consumption of tomato protects against cancers of digestive tract, stomach, colon and rectum and this beneficial effect is due to the presence of antioxidant compounds like lycopene, carotenoids and vitamin C. It is a very good appetizer and remedy for patients suffering from constipation, if used as a soup (Hazara *et al.,* 2009).

The tomato growing areas are facing threat from several diseases such as bacterial wilt, damping off, *Fusarium* wilt, early blight, late blight, mosaic, leaf curl and so on. Among the various diseases affecting the crop, bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* is the major one and causes extensive losses in Asia and South Pacific regions (Persley, 1986). The pathogen is soil-borne with a wide host range and the disease occurs in diverse soil types of acidic and alkaline nature. It affects solanaceous vegetable crops in Kerala, which is having a tropical humid climate conducive for the incidence of the disease. The yield loss due to bacterial wilt in tomato ranges from 10.80 – 92.62% in India (Ramkishun, 1987).

The control of bacterial wilt disease with chemicals is not effective due to the variability in bacterial wilt pathogen and its wide host range. Moreover, indiscriminate use of chemicals results in environmental pollution, development of resistant strains, detrimental effects on a variety of non target organisms and also affects the human health due to the direct consumption of vegetables. So,

biological control of plant disease is an alternative approach due to its eco-friendly nature, cost effectiveness and long lasting effects.

Biocontrol agents include rhizospheric organisms which are seen especially near the rhizosphere of plants and endophytic organisms that resides within a plant. 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). Endophytes are known to benefit the host by promoting plant growth and also prevent pathogenic organisms from colonization. Endophytic habitat appears to provide a protective environment that helps a potential endophyte with reduced competition from the indigenous microbial population. Franco *et al.* (2006) noticed that endophytic actinomycetes are effective biocontrol agents as they influence plant growth through enhanced germination, production of growth hormones and as bioagents mediated through antibiotic production and also by inducing plant systemic defense pathways.

So far, no work been has been carried out in India on the effect of endophytic actinomycetes on plant growth promotion and bacterial wilt disease management. Hence, this research project was undertaken to study the bioeffficacy of endophytic actinomycetes on plant growth promotion and management of bacterial wilt in tomato with the following objectives:

- To study the antagonistic effect of endophytic actinomycetes against bacterial wilt pathogen under *in vitro* conditions
- To evaluate promising endophytic actinomycetes against bacterial wilt pathogen under pot culture conditions.

Review of literature

2. REVIEW OF LITERATURE

Tomato is one of the most popular protective foods because of its high lycopene content and is a widely grown vegetable in the world. It is a rich source of vitamins and minerals. They are consumed as fresh or in the processed form. Being versatile for culinary purposes, it is also one of the most commonly grown vegetable in the kitchen garden (Rana, 2008).

2.1. BACTERIAL WILT IN TOMATO

Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al.* is one of the most destructive diseases of tomato in the warm humid regions of the world. The occurrence of bacterial wilt in tomato was first reported by Hedayathullah and Saha (1941) from West Bengal. Later, it was studied on brinjal by Das and Chathopadhyay (1955), on banana by Chathopadhyay and Mukhopadhyay (1968) from West Bengal and on chillies by Remadevi (1978) from Kerala.

The disease is prevalent in temperate, tropical and sub-tropical regions of the world (Kelman, 1954). Das and Chathopadhyay (1955) studied in detail about bacterial wilt of brinjal and estimated an average reduction in yield of 54.6 to 62.3 per cent due to this disease. The vegetable growers are suffering from heavy losses every year due to this disease and the cultivation of solanaceous vegetables is getting restricted. Rao and Sohi (1977) estimated that in India there was 25-75 per cent yield loss in solanaceous vegetables due to bacterial wilt disease. Ramkishun (1987) reported that the loss in yield due to the disease in tomato ranges from 10.80 – 92.62% in India. Elsewhere, the losses as to the tune of $ 496000/ha during June-October has been reported from Taiwan (Hartman *et al.*, 1991). According to Shekhawat *et al.* (1992), plant species belonging to 37 families in India were attacked by bacterial wilt pathogen. Sumithra *et al.* (2000) conducted a field survey in Karnataka and observed that bacterial wilt of egg plant

was wide spread in the state. Osiru *et al.* (2001) assumed that bacterial wilt was a serious and devastating disease limiting tomato production worldwide.

Ralstonia solanacearum has a wide host range and race differentiation which makes it difficult to identify the pathogen and control the disease. Buddenhagen *et al.* (1962) observed race 1 of the pathogen occurring in tropical areas and affecting tomato, tobacco, other solanaceous crops and a few diploid bananas. Race 2 occurred mainly in tropical areas of South America attacking banana and heliconia. Race 3 occurred at higher altitudes in the tropics, sub tropics and temperate areas attacking potato and tomato and was weakly pathogenic to other solanaceous crops. Race 4 was particularly aggressive on ginger and race 5 was specialized on *Morus*. Keshwal and Joshi (1976) studied isolates of *Pseudomonas solanacearum* E.F.Smith and found that the isolate G3173 could infect ageratum, tomato and brinjal, but no other solanaceous hosts.

Rath and Addy (1977) noted ten isolates causing wilt of tomato belonging to race 1. After studying different isolates from many parts of Kerala, Remadevi (1978) reported that *R.solanacearum* exists in different races or strains coming under race 1 or race 3. Two isolates from Vellayani, Thiruvananthapuram were found highly virulent on tomato, brinjal or chilly. This indicated that the same strain of the pathogen could infect and produce disease in more than one host in different locations. It was also inferred that different pathotypes of *R.solanacearum* varying in pathogenecity and also in cultural, physiological and biochemical properties were present in Kerala soils. Der and Hsein (1999) demonstrated bacterial wilt in some, but not all ecotypes of *Arabidopsis thaliana*, were caused by strains of *R.solanacearum*. Khan *et al.* (1999) identified the causal organism of bacterial wilt in tobacco as *R.solanacearum* by fatty acid profiles and polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) analysis. Physiological and biochemical test also confirmed this conclusion.

Sharma and Rana (1999) observed that bacterial wilt was present 80% of 310 ginger fields surveyed in Himachal Pradesh. Hung *et al.* (2000) observed the occurrence of bacterial wilt of jute in some fields in Central Taiwan. Based on cultural characteristics, physiological and biochemical properties, biolog system and pathogenecity test, the causal organism was identified as *R.solanacearum.* The isolate obtained in this study was race 1 and biovar 4. The pathogen could induce wilting not only on jute but also on other solanaceous plants. Another recent classification of *R.solanacearum*, based on RFLP and other genetic fingerprinting studies was in to Division I (biovars 3, 4, and 5 originating in Asia) and Division II (biovars 1, 2A and 2T originating in South America) (Hayward, 2000).

Dookum *et al.* (2001) determined the genetic diversity among *R.solanacearum* strains isolated from potato, tomato, beans and anthurium using RFLP techniques. Mathew (2001) and Mathew *et al.* (2002) reported biovar 3, 3A and 5 of tomato isolates of *R.solanacearum* from different locations of Thrissur and Palakkad districts of Kerala. James (2001) reported biovar 3 from Kumarakom and Ambalavayal and biovar 3A from Vellanikkara. She also reported that isolates of Vellanikkara and Kumarakom belonged to race 1, whereas the Ambalavayal isolates belonged to race 3.

2.2. ISOLATION AND MAINTENANCE OF PATHOGEN

2.2.1. Isolation of *R.solanacearum*

A medium for the isolation and detection of *P.solanacearum* was developed by Kelman (1954) by adding triphenyl tetrazolium chloride (TZC) to differentiate weakly pathogenic or avirulent mutants from fluidal wild type. Normal or wild type formed an irregularly round, fluidal white colony with a pink centre while the mutant formed a round, butyrous deep red colony with a narrow bluish border. The same method was followed by several other workers for the isolation of

P.solanacearum (Hussain and Kelman, 1958; Khan *et al.*, 1979; Nayar, 1982; He *et al.*, 1983; Swanepoel and Young, 1988; Prior and Steva, 1990; Jyothi, 1992; Paul, 1998; Ito, 1998 and James, 2001). TZC formed the basic component of many selective media for the isolation of *P.solanacearum* which were developed later. Karganilla and Buddenhagen (1972) developed a selective medium consisting of some antimicrobial compounds which reduced the background population by 90%, but the population of *P.solanacearum* was also reduced by 22 to33%. Nesmith and Jenkins (1979) developed a new selective medium by adding the antimicrobial compounds at the time of isolation and the colonies of *P.solanacearum* were similar in appearance to those observed on TZC.

Chen and Echandi (1982) developed TZC CP medium by adding chloramphenicol (10 µg/ml) to the TZC basal medium which was effective by reducing 75-95% contamination by other bacteria and fungi. But the detection of *P.solanacearum* with this medium was not satisfactory as it required 24 hours more incubation as compared to the TZC medium and by this time the plates got contaminated with other bacteria and fungi. Granada and Sequeira (1983) developed SM1 and SM2 media to isolate *P.solanacearum* from artificially and naturally infested soils. Antimicrobial compounds such as crystal violet, thimerosal, polymixin B sulfate, tyrothricin and chloromycetin were used to reduce bacterial contamination and cycloheximide or chlorothalonil were added to reduce bacterial contamination. The plating efficiency here ranged from 80-100% of that on TZC medium.

Engelbrecht (1994) compared a modified form of a selective medium developed by Graham Lloyd (SMSA) with the original medium (GL) and SM1 medium. He found GL as efficient but failed to reduce soil saprophyte contamination. Hara *et al.*(1995) developed the improved Hara and Ono's medium (HOM) and named PCCG which showed higher sensitivity to *R.solanacearum*. This PCCG medium was later used for the detection of viable

cells of *R.solanacearum*. It was easy to distinguish the fluidal colonies from the butyrous colonies on CCG medium.

2.2.2. Maintenance of *R.solanacearum*

It is essential to maintain the bacterial cultures after the isolation for further studies. *R.solanacearum* was earlier maintained on PDA slants and covered with sterile mineral oil (Kelman and Jenson, 1951; Winstead and Kelman, 1952). The culture remained viable for four years. Hussain and Kelman (1958) maintained stock cultures by suspending five loopful of bacterial culture in 5 ml of sterile distilled water and storing at 25^0 C. This method was followed by some other workers also (Kelman and Person, 1961; Khan *et al.*, 1979; Granada and Sequeira, 1983; Swanepoel and Young, 1998; Prior and Steva, 1990; Mathew *et al.*, 2000). Kumar *et al.* (1993) maintained cultures under refrigerated conditions at 4^0 C.

2.3. CONCEPT OF ENDOPHYTES

The word 'endophyte' is derived from two Greek words, 'endon' meaning 'within' and 'phyton' meaning 'plant'. 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 lifecycle 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).

Common endophytes include a variety of bacteria, fungi and actinomycetes which can be isolated from wild or cultivated crops (Liu and Tang, 1996) of either the monocotyledonous or dicotyledonous plant groups (El-Shanshoury *et al.*, 1996). According to Schulz *et al.* (1999), endophytes are widely assumed to be present in virtually all land plants. Holderness *et al.* (2000) also reported that endophytic microorganisms are present in various plant species and rarely produce any symptoms of disease. In many cases the endophytic- host association has been viewed as a mutualistic association (Saikokken *et al.*, 2004) and these endophytes can have many effects on their host such as enhancement of stress resistance, insect and disease resistance and productivity improvements. The colonization and propagation of endophytes and their secondary metabolites inside the host plant may be critical for these effects (Tian *et al.*, 2004).

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.*, 2007). Shiel *et al.* (2009) reported that endophytes are important in epidemiology because certain endophytic associations lead to the enhancement of pathogen resistance in plants and an increase in vegetative growth when compared to similar uninfested plants.

2.4. ENDOPHYTIC ACTINOMYCETES

Actinomycetes are gram positive, filamentous and primarily soil inhabiting microbes. The name of the group is derived from two words, 'actinos' meaning 'ray' and 'mykes' meaning 'fungus' (Sullia and Santharam, 2005). Actinomycetes are very widely distributed and they produce medically useful natural antibiotics. They can degrade a variety of organic compounds and are extremely important in the mineralization of organic matter. Although, most of them are free living and endophytes, a few are pathogenic to humans, other animals and some plants

(Purohit, 2006). Morphologically, actinomycetes fall between fungi and bacteria. Their filamentous nature, branching pattern and conidia formation are similar to those of fungi. But their size and spore characters are similar to bacteria. Recent detailed studies have brought out clearly that actinomycetes are quite distinct from bacteria and fungi in many respects. They produce a mass of growth to form a colony, but that differs from a bacterial colony in the sense that it consists of filamentous threads with spore bearing hyphae (Rangaswami and Bhagyaraj, 2007).

The endophytic presence of actinomycetes plays an important role in plant development and health because they can affect plant growth by nutrient assimilation or by secondary metabolite production (Kunoh, 2002). Castillo *et al.* (2002) reported that endophytic actinomycetes have been isolated from stem and root interior of many plants such as tomato, banana and wheat. Compared to actinomycetes in the rhizosphere, endophytic actinomycetes are not subjected to competition from soil bacteria and can colonize plants well and so they can be considered as potential biocontrol agents (Cao *et al.*, 2004a).

According to Cao *et al.* (2005), the actinomycetes which resided inside healthy plant tissues without causing any symptoms of disease were defined as 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*. Franco *et al.* (2006) noticed that endophytic actinomycetes are effective biocontrol agents as they influence plant growth through enhanced germination, production of growth hormones and as bioagents mediated both through antibiotic production and also by priming plant systemic defense pathways. 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 pathogenic strains have rapidly acquired resistance.

2.5. ISOLATION OF ENDOPHYTIC ACTINOMYCETES

The isolation procedure of endophytic actinomycetes is of key importance for research with them. Sardi *et al.* (1992) reported the presence of actinobacteria in the root samples of crops and Italian native plants with the majority belonging to the genus *Streptomyces.* Okazaki *et al.* (1995) were able to isolate *Microbiospora* sp. at a much higher frequency from plant leaves than from soil. de-Aurajo *et al.* (2000) found that actinobacteria could be isolated from the roots and leaves of maize on starch- casein- agar medium and 2.5% water agar medium.

To survey the potential of endophytic actinomycetes as bioagents against fungal pathogens of rhododendron, Shimizu *et al.* (2000) placed surface sterilized young plants of rhododendron in IMA-2 agar medium. Nine, six and two isolates were obtained from root, stem and leaves respectively suggesting that various species of actinomycetes grow in plants as symbionts or parasites. Taechowisan and Lumyong (2003) isolated 59 endophytic actinomycetes from the roots of *Zingiber officinale* and *Alpinia galanga* by transferring surface sterilized tissue pieces to dishes of humic acid- vitamin agar (HV) agar containing 100 µg/ml nystatin and cycloheximide and incubated at 30^0 C for one month. Coombs and Franco (2003) isolated filamentous actinomycetes from surface sterilized root tissues of healthy wheat plants from a range of sites across South Australia.

Tian *et al.* (2004) studied the populations of endophytic fungi and actinomycetes from rice cultivars of Panayu and Wushan districts of South China. More diverse endophytic actinomycetes were isolated from roots rather than from leaves. Ten fragments from different parts of leaves and roots per rice sample were placed on 1.2% S medium supplemented with 25 ppm potassium dichromate and 15 ppm nalidixic acid for the isolation. Cao *et al.* (2004a) successfully isolated 240 actinomycetes strains from the interior of leaves and roots of healthy and wilting banana plants by keeping small fragments of the samples on S

medium with 15µg nalidixic acid/ml to suppress the growth of bacteria and incubated at 26^0 C. Cao *et al.* (2004b) isolated endopophytic *Streptomyces* from the roots of tomato and studied their use as potential biocontrol agents against the pathogen *Rhizoctonia solani*. A total of 131 endophytic actinomycetes strains were successfully isolated from surface sterilized banana roots using S medium (Cao *et al.,* 2005). Tan *et al.* (2006) isolated endophytic actinomycetes from healthy and wilting tomato grown in three different sites of South China and studied their activities against *Ralstonia solanacearum* under *in vitro*. Most of the isolates belonged to *Aureus* group of *Streptomyces*. The proportion of antagonistic strains from healthy plants was higher than that from wilting plants. The population composition of antagonistic *Streptomyces* varied according to tomato cultivars, physiological status and soil types.

Teng *et al.* (2006) reported that endophytic actinomycetes RS4 (*Chrysobacterium* sp.), RS65 and RS70 (*Streptomyces* sp.) isolated from tomato plants were better in their ability to manage bacterial wilt. Zhiqi *et al.* (2006) isolated 58 actinomycetes from surface sterilized tomato roots and they were screened for their herbicidal activities. Jiefeng *et al.* (2009) were able to isolate 44 endophytic actinomycetes from three solanaceous vegetables such as tomato, pepper and egg plant. Verma *et al.* (2009) isolated 55 endophytic actinomycetes from *Azadirachta indica* using S- agar medium and 2.5% water-agar medium. Of the 55 isolates 54.5% were recovered from roots followed by stem (23.6%) and leaves (21.8%).

Another most common procedure for the isolation of endophytes were based on trituration of surface disinfested plant tissues using disinfectants like sodium hypochlorite (Fisher *et al.,*1992), mercuric chloride (Sriskandarajah et al., 1993), hydrogen peroxide (Mclnroy and Kloepper, 1994) or a combination of of two or more of these disinfectants (Pleban *et al.,* 1995). After surface disinfestations, the plant tissues were triturated with a pestle and mortar in sterile water, buffer solutions or a liquid media and then the endophytes were enumerated from the

triturate. To decrease the possibility of recovery of surface contaminants, sterility checks should be included either by dipping roots in nutrient broths (Gange *et al.*, 1987) or by transferring 0.1 ml of the final washing solution to a test tube with medium (Mclnroy and Kloepper, 1994). According to Hallmann *et al.* (1997), trituration technique can be used for almost all plant tissues including flowers, fruits and seeds.

2.6. IDENTIFICATION OF ENDOPHYTIC ACTINOMYCETES

The two main approaches for the identification of endophytic actinomycetes can be grouped into classical approach and the molecular approach.

2.6.1. Classical approach

Classical approach makes use of morphological and cultural characters for the identification of actinomycetes. The classical method described in the identification key by Nonmura (1974) and Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) is very much useful for the identification purposes. Cultural and morphological characteristics including presence of substrate and aerial mycelia, spore mass color, distinctive reverse colony color, color of diffusible pigments, sporophore and spore chain morphology were used as the identification characters and were studied after 10-14 days of incubation on various International Streptomyces Project (ISP) media (Shirling and Gottlieb, 1966; Ruan *et al.*, 1990; Sardi *et al.*, 1992; Yan, 1992; Shimizu *et al.*, 2000; Zheng *et al.*, 2000; Nishimura *et al.*, 2002; Taechowisan and Lumyong, 2003; Tian *et al.*, 2004; Cao *et al.*, 2004a; Cao *et al.*, 2004b; Cao *et al.*, 2005; Tan et al., 2006; Verma *et al.*, 2009).

2.6.2. Molecular approach

The most powerful approaches to taxonomy of microbes are through the study of nucleic acids. Because these are either direct gene products or the genes themselves and comparisons of nucleic acids yield considerable information about relatedness. Molecular systematics which include both classification and identification has its origin in the early nucleic acid hybridization techniques, but achieved a new status following the introduction of nucleic acid sequencing techniques (O'Donnell *et al.,* 1993). Significance of phylogenetic studies based on 16S rDNA sequencing is increasing in the systematic of actinomycetes (Yokota, 1997). Sequences of 16S rDNA have provided actinomycetologists with a phylogenetic tree that allows the investigation of evolution of actinomycetes and also provides the basis for identification. Analysis of 16S rDNA begins by isolating DNA and amplifying the gene coding for 16S rRNA using the polymerase chain reaction. The 30S subunit of actinomycetes ribosome has 16S rRNA and it is used to find out the degree of similarity or relatedness between two species, because it has been found to remain relatively stable during evolution (Purohit, 2006).

Wellington *et al.* (1992) were able to identify four species classified within the genus *Kitasatosporia* using hybridization with a 16S rRNA genus probe for *Streptomyces* sp. indicating a close relationship within the two genera. To study the ecology of filamentous nocardiform actinomycetes at the molecular level, Schuppler *et al.* (1995) established a clone library of 16S rRNA gene fragments from the sludge DNA. Chun and Goodfellow (1995) studied the partial sequences of 16S rRNA genes of the type strains of nine species of the genus *Nocardia* following the isolation and cloning of amplified genes. Heuer *et al.* (1997) analysed actinomycetes communities by specific amplification of genes encoding 16S rRNA and gene electrophoretic separation in denaturing gradients. A group specific primer, f243 was developed by comparison of sequences of genes encoding 16S rRNA for the detection of actinomycetes in the environment with PCR.

Laurent *et al.* (1999) reported that the 16S rRNA analysis offers a specific and rapid alternative to chemotaxonomic method for the identification of *Nocardia* sp. of clinical importance isolated from pathogenic samples. Conville *et al.* (2000) evaluated the usefulness of PCR amplification of a portion of the *Nocardia* 16S rRNA gene and subsequent restriction endonuclease analysis for species identification. Hall *et al.* (2001) identified clinical isolates of actinomycetes by 16S rRNA restriction analysis. Taechowisan and Lumyong (2003) observed that 16S rRNA gene sequencing of isolates from the roots of *Zingiber officinale* and *Alpinia galanga* against phytopathogenic fungi revealed that the strains were phylogenetically closely related to *Streptomyces aureofaciens.* 16S rRNA was amplified by PCR using primers, A 7-26f and B 1523-1504r. Coombs and Franco (2003) isolated 49 actinomycetes from surface sterilized wheat roots and were identified using 16S rRNA sequencing and found that isolates belong to group of genera including *Streptomyces, Microbiospora, Micromonospora* and *Nocardia* sp.

A rapid method for the identification of filamentous actinomycetes genera was developed based on 16S rRNA gene restriction fragment patterns by Cook and Meyers (2003). The patterns were generated using specific restriction endonucleases to perform *in silico* digestions of the 16S rRNA gene sequences of all validly published filamentous actinomycetes. Nearly full length of 16S rDNA sequences were amplified using primers F1 and R5. Primer F1 binds to the base positions 7-26 and primer R5 to the base positions 1496-1476 of the 16S rRNA gene of *Streptomyces ambofaciens.* Patel *et al.* (2004) investigated the utility of 500bp 16S rRNA gene sequencing for identifying aerobic actinomycetes genera such as *Streptomyces and Nocardia.* Cao *et al.* (2004a) analysed more than 500bp of 16S rRNA genes BLASTN confirmed 99% similarity between the strains S76, which was isolated against *Fusarium oxysporum* f.sp. *cubense* of banana as *Streptomyces griseorubiginosus.* Cheng-Ling *et al.* (2008) isolated antagonistic actinomycetes from soil samples collected from ginger field against *Ralstonia solanacearum.* They were identified by 16S rRNA sequence analysis which

revealed that they belonged to *Streptomyces lavendulae.* Jeffrey (2008) reported that 16S rRNA sequencing of 62 actinomycetes isolated against *R.solanacearum* from seven soil samples collected from Agricultural Research Centre, Semongok revealed that they belong to *Streptomyces* sp. Nimnoi *et al.* (2010b) used PCR-denaturing gradient gene electrophoresis to determine diversity and community of endophytic actinomycetes distributed within the roots of *Aquilaria crassna* Pierre ex Lec (eagle wood). PCR-DGGE analysis of a variable region of 16S rRNA confirmed the presence of endophytic actinomycetes in the genera *Nocardia, Pseudonocardia, Streptomyces* and *Actinomadura* within the roots of eaglewood.

2.7. ENDOPHYTIC ACTINOMYCETES AS BIOCONTROL AGENTS

2.7.1. Concept of biological control

Biological control can be defined as the reduction in the amount of inoculum or disease producing activity of a pathogen accomplished by or through one or more organisms other than man (Cook and Baker, 1983; Baker 1987). Since 1960's there has been a remarkable increase of interest and research on biological control because of various concerns about environmental pollution, development of pesticide resistance in pathogens and lack of adequate resistance in crops to many of the pathogens. Biocontrol agents fit well with organic farming which is gaining importance in recent years (Harman *et al.,* 1989). Biocontrol agents gets stabilized once efficient strains that fit into the concerned ecological niche are introduced into an environment in contrast to agrochemicals which get leached off during incessant rains. Thus, biological control reduces the dependence on high risk chemicals for disease management and is ecologically sound and environment friendly (Bowen and Rovira, 1999).

2.7.2. Mechanism of action of endophytic actinomycetes as a biocontrol agent

Understanding the diversity of mechanisms of biological control will lead to the improved application of different strains as a bioagent. The various mechanisms are complex and what has been identified as biological control is the final result of different mechanisms acting synergistically to achieve the disease control (Howell, 2003). The activity of biocontrol agents depend mainly on the different physico chemical environmental conditions to which they are subjected (Benitez *et al.*, 2004). The association of endophytic actinomycetes with plants is found to confer protection against plant pathogens through various mechanisms like production of antibiotic substances, cell wall degrading enzymes, siderophores and volatile- non volatile metabolites (Clegg and Murray, 2002; Bailey *et al.*, 2006).

2.7.3. Antibiosis

Antibiosis is one of the most important mechanisms of biocontrol. It plays an active role in the biocontrol of plant diseases and often acts in concert with competition and parasitism. Antagonists may produce powerful growth inhibitory compounds that are effective against a wide array of microorganisms and such compounds are referred to as antibiotics (Ownley and Windham, 2003). Strobel and Daisy (2003) defined antibiotics as low-molecular weight organic compounds produced by microbes which are active at low concentration against other microbes. Often endophytes are potential sources of these antibiotics and natural products from endophytic microorganism have been observed to inhibit or kill a variety of harmful disease causing agents. Cao *et al.* 2005 reported that the use of endophytic actinomycetes as biocontrol agents is of interest through their ability to colonize healthy plant tissues and produce antibiotics *in situ*.

Antibiosis is determined by pairing on agar plates (Looper and Chilton, 1950; Jhonson and Curl, 1972). There have been several modifications of this method including Herr's triple agar- layer plate technique (Herr, 1959), agar-ring method (Williams and Wills, 1962), reversed layer method (Hasegawa *et al.*,

1990), all of which give an indication of the level of effectiveness of the antagonists to inhibit the vegetative growth of the pathogen. These are usually followed by test involving culture filtrates or purified fractions of the filtrates (Smith, 1957; Rothrock and Gottlieb, 1984; Upadhyay and Rai, 1987; El-Abyad *et al.*, 1993; Yuan and Crawford, 1995; Trejo-Estrada *et al.*, 1998b).

Gurney and Mantle (1993) detected 1-N- methylalbonoursin of antifungal activity in the culture of endophytic actinomycetes species isolated from perennial rye grass. Beiber *et al.* (1998) reported a napthoquinone antibiotic, alnomycin in *Streptomyces* sp. isolated from the root nodules of *Alnus glutinosa* collected in Germany. Igarashi *et al.* (2000) found that one of the extracts from the *Streptomyces* sp. isolated from *Allium fistulosum* had a potential to suppress the infection of *Alternaria brassicola* on Chinese cabbage seedlings. Such an effect was dependent on a novel plant protective compound named fistupyrone. Their results suggested that a wide range of endophytic actinomycetes have a potential to produce antimicrobial compounds which probably contribute to the competition with other microbes in host plants.

Sasaki *et al.* (2001a&b) identified several new bioactive compounds produced by actinomycetes isolated from live plants. Two new novobiocin analogues were produced by *Streptomyces* sp. collected from *Aucuba japonica* and cedarmycins by *Streptomyces* sp. collected from *Cryptomeria japonica* and were determined to be antimicrobial metabolites. Castillo *et al.* (2002) reported that *Streptomyces* sp NRRL30562 obtained from snake vine plant was proved to produce novel peptide antibiotics designated as munumbicins and they possessed a wide spectrum activity against phytopathogenic bacteria and fungi. Igarashi *et al.* (2002a) isolated 398 actinomycetes strains from leaves, stem and roots of cultivated and wild plants. About 10-20% of the n-butanol extracts of their fermentation broths showed antagonistic activity against phytopathogenic bacteria and fungi.

El-Tarabily (2003) observed that crude culture filtrate of *Actinoplanes missouriensis* exhibited antifungal activity against the pathogen *Plectosporium tabacinum,* the causal agent of lupin root rot. Castillo *et al.* (2003) found that *Streptomyces* sp. NRRL30566 which was isolated from a fern leaf, *Greviella pteridifolia* growing in the northern territory of Australia produced wide spectrum antibiotics named kakadumycins. Shimizu *et al.* (2004) proved that *Streptomyces galbus* strain R5 isolated from leaves, stem and root of produced antibiotics like actinomycin X_2 and fungichromin that could account for its *in vitro* antibacterial and antifungal activities. Erza *et al.* (2004) noted that coronamycin a peptide antibiotic isolated from the culture broth of *Streptomyces* sp. MSU 2110 recovered from the stems of *Monstera* sp. exhibited antagonistic activity against phytopathogenic *Pythium* sp.

Taechowisan *et al.* (2005) reported that *Streptomyces aureofaciens* strain CMUAC 130 was isolated from the root tissue of *Zingiber officinale.* It was an antagonist of *Colletotrichum musae* and *Fusarium oxysporum* f.sp. *cubense,* the causative agents of anthracnose and wilt in banana. The culture filtrate and crude extract from this strain were inhibitory to the tested phytopathogenic fungi. The major active ingredients from the culture filtrate of *S.aureofaciens* CMUAC 130 were purified by silica gel column chromatography and identified to be 5,7-dimethoxy- 4-p-methoxyl phenyl coumarin and 5,7 dimethoxy-4-phenyl coumarin by NMR and mass spectral data respectively. Bioassay studies showed that these compounds had antifungal activities against the tested fungi.

Ya-Ping *et al.* (2007) observed that 29 strains of endophytic actinomycetes were isolated from surface sterilized plant tissues of wild plants collected from China. The fermentation filtrates of their metabolites exhibited greater antagonism against *Alternaria solani.* According to Zhe *et al.* (2008), active metabolites from endophytic actinomycetes presented an inhibiting activity to the pathogen, *Pseudomonas syringae* pv *actinidae* causing kiwi fruit bacterial canker.

2.7.4. Production of cell wall degrading enzymes

Actinomycetes secrete hydrolytic enzymes such as cellulases, hemi cellulases, chitinases, amylases and glucanases which degrade the cell wall of pathogen (Geetha and Vikineswary, 2002). El-Tarabily (2003) isolated 21 streptomycetes and 15 non-streptomycetes actinomycetes from surface sterilized lupin roots and evaluated their potential to produce chitinase and to inhibit the growth of *Plectosporium tabacinum*, the causal agent of lupin root rot in Egypt. The most inhibitory isolate was identified as *Actinoplanes missouriensis*, which produced relatively high levels of chitinase and degraded the hyphae of *P. tabacinum* under *in vitro* conditions causing extensive plasmolysis and cell wall lysis. Taechowisan *et al.* (2003).screened more than 300 isolates of endophytic actinomycetes for their potential to produce chitinase. *Streptomyces aureofaciens* strain CMUAC 130 was the most effective producer of chitinase. The production of chitinase was optimal 1% colloidal chitin concentration and also the addition of pectin, starch and carboxyl methyl cellulose (CMC) to colloidal chitin containing medium increased chitinase production. The crude or purified enzyme had potential for cell wall lysis of many phytopathogenic fungi tested.

Tan *et al.* (2006) reported that endophytic actinomycetes isolated from stem and roots of tomato plants showed the ability to degrade the cell wall of the bacterial wilt pathogen *R.solanacearum*. El-Tarabily and Sivasithamparam (2006) reported that among the lytic enzymes, chitinases have been studied largely because these enzymes are produced by a variety of endophytic microorganisms. Quecine *et al.* (2008) evaluated chitinase production by endophytic actinomycetes. Endophytic *Streptomyces* were grown on minimal medium supplemented with chitin and the chitinase production was quantified. The strains were screened for their activity against phytopathogenic oomycetes by a dual culture *in vitro* assay and the correlation between chitinase production and pathogen inhibition was calculated.

El-Tarabily *et al.* (2009) reported endophytic glucanases producing actinomycetes namely *Actinoplanes companulatus, Micromonospora chalcea* and *Streptomyces spirales*. They protected seedlings and mature plants of cucumber from diseases caused by *Pythium aphanidermatum* under greenhouse conditions. Ning *et al.* (2010) reported that *Streptomyces longisporflavus* showed high inhibition efficiency of 85.4% to the mycelia growth of *Sclerotina sclerotiorum* by the production of chitinases. El-Tarabily *et al.* (2010) reported that the success of the endophytic actinomycetes, *Streptomyces spiralis* over *Actinoplanes companulatus* in the control of *Pythium aphanidermatum* of cucumber under commercial field production in UAE was due to its ability to produce higher levels of β-1, 3; β-1, 4 and β-1, 6 glucanases. Nimnoi *et al.* (2010) reported that 10 endophytic actinomycetes isolated from healthy shoots and roots of *Aquilaria crassna* (eagle wood) showed protease activity ranging from undetectable to 8.16 units/ml.

2.7.5. Production of siderophores

The term 'siderophore' is a Greek word meaning "iron bearers". They are low molecular weight (0.5 to 1.5 kDa) and highly specific iron chelating agents; which can compete for ferric iron in ferric hydroxide complexes. They are produced under iron limiting conditions by almost all aerobic and facultative anaerobic microorganisms. These iron ligands facilitate the translocation of oxidized iron ions across the lipid bi-layer of the cytoplasmic membrane and supplies to the cell (Nielands, 1981).

Iron is an essential nutrient for all living cells since it is required in oxidation- reduction and other important cellular reactions which involve the participation of cytochromes, ferredoxins, nitrogenases and ribonucleotide reductases (Halliwell and Gutteridge, 1986). Iron limitation poses a serious threat to microorganisms as they are unable to survive without adequate supply of iron. Siderophores selectively chelates iron and make it unavailable to soil borne

pathogens and other deleterious microorganisms thus reducing their population (Schippers *et al.*, 1987). Nielands (1995) also reported that siderophores are produced under conditions of low iron availability. Cao *et al.* (2005) observed that limited availability of iron in the soil could favor suppressiveness to *Fusarium* wilt. Siderophores produced by endophytic *Streptomyces* strain may induce soil suppressiveness when ferric iron is relatively low in concentration in the soil. Tan *et al.* (2006) reported that the percentage of siderophores producing isolates varied when they were isolated from the same tomato cultivars collected from different sites. The results suggested that the different soil environments of each site probably contained different concentrations of available iron and also the ability of endophytic *Streptomyces* isolated from different sites to absorb available iron varied. Khamna *et al.* (2009) reported that *Streptomyces* sp. is known for the production of hydroxamate type of siderophores, which inhibit the phytopathogens growth by competing for iron in the rhizosphere soils.

Nimnoi *et al.* (2010) observed that among 10 endophytic actinomycetes isolated from healthy shoots and roots of *Aquilaria crassna* (eagle wood), eight isolates produced hydroxamate type of siderophores ranging from 3.21 µg/ml to 39.30 µg/ml. Only one isolate, *Actinomadura glauciflava* produced catechol type of siderophores in the amount of 4.12 µg/ml. de-Oleviera *et al.* (2010) reported that of the 70 endophytic actinobacteria isolated from tomato plants in Southern Brazil, 86.8% produced siderophores. Misk and Franco (2011) noted that of the 11 endophytic actinomycetes isolated from plants such as lentil, chickpea, bean and wheat, 72% produced siderophores.

2.7.6. Production of volatile and non-volatile metabolites

Volatile substances are organic or inorganic compounds which are low in molecular weight and also in polarity, but high in vapor pressure. Production of volatile substances has been reported in *Streptomyces* sp. and other related genera by many workers (Collins and Gains, 1964; Gerber and Lechevalier, 1965;

Moore-Landecker and Stotzky, 1973; Gerber, 1973; Herrington *et al.*, 1985; Carpenter-Boggs *et al.*, 1995; Scholer *et al.*, 2002; Dickschat *et al.*, 2005; Vesperman *et al.*, 2007). Moore-Landecker and Stotzky (1973) reported that volatile substances from an actinomycete caused abnormal shortening of conidiophores in *Aspergillus giganteus* and vesicle like swellings in *Fusarium oxysporum, Penicillium viridicatum* and *Trichoderma viridae.* Herrington *et al.* (1985) noted that methyl vinyl ketone, a volatile metabolite produced by *Streptomyces griseoruber* could inhibit the spore germination of *Cladosporium* sp. El-Tarabily *et al.* (1997) screened actinomycetes isolates from carrot rhizosphere for their *in vitro* and *in vivo* antagonism to *Pythium coloratum*, a causal agent of cavity spot disease in carrots. Of the total isolates, seven isolates including streptomycetes and non-streptomycetes actinomycetes tested produced non volatile antifungal metabolites.

Blumer and Hass (2000) reported that cyanide is an important volatile substance produced by endophytes and it is formed as a secondary metabolite. It is assumed that its formation has an advantage for the organism by inhibiting other competing microbes. Wan *et al.* (2008) observed that volatile substances from *Streptomyces platensis* isolated from a healthy plant of rice suppressed the growth of *Sclerotina* leaf blight of rape, *Botrytis* fruit rot of strawberries, Rhizoctonia leaf and seedling blight of rice. Results showed that 16 volatile substances were released from cultures of *Streptomyces platensis*. These compounds fell into classes of alcohols, esters, organic acids, alkanes, ketones and alkenes.

Macagnan *et al.* (2009) noted that inhibition of *Crinipellis perniciosa*, the causal agent of cocoa witches broom is due to the production of hydrogen cyanide by five actinomycetes belonging to the genus *Streptomyces.* Nimnoi *et al.* (2010) observed that among 10 endophytic actinomycetes isolated from healthy shoots and roots of *Aquilaria crassna* (eagle wood), produced ammonia ranging from 2 to 60 mg/ml. Misk and Franco (2011) noted that of the 11 actinomycetes isolated

from plants such as lentil, chickpea, bean and wheat from South Australia, 33% were positive for cyanogens production.

2.8. EFFECT OF ENDOPHYTIC ACTINOMYCETES ON PLANT DISEASE MANAGEMENT

One of the reasons behind the use of endophytic actinomycetes as biocontrol agents in plant disease management is due to their ability to colonize healthy plant tissues and produce a wide range of bioactive secondary metabolites such as antibiotics which possesses both antibacterial and antifungal activities. El-Abyad *et al.* (1993) reported three *Streptomyces* sp. having potential of microbial antagonism for the control of some tomato diseases including bacterial wilt. *In vitro* studies showed that 80% culture filtrate of either *S.pulcher* or *S.citreofluorescens* was detrimental to the bacterial population of *R.solanacearum*. Moura *et al.* (1998) isolated endophytic actinomycetes from the root tissues of tomato and it was assessed against *R.solanacearum*. Out of the total isolates, 18 isolates showed 100% control of the pathogen.

Moura and da-Romeiro (1999) assessed 190 actinomycetes isolates against 52 isolates of *R.solanacearum* from different regions of Brazil and worldwide from various hosts. Only a few actinomycetes showed no activity against the pathogen, a great number had intermediate activity and a few showed high activity following a normal distribution. Shimizu *et al.* (2000) surveyed the potential of endophytic actinomycetes as biocontrol agents against fungal pathogens of rhododendron. A *Streptomyces* isolate R5, formed a clear growth inhibition zone against two major fungal pathogens, *Phytophthora cinnamomi* and *Pestalotiopsis sydowiana* indicating that the isolates can produce antifungal metabolites. Nishimura *et al.* (2002) observed that *Streptomyces* sp. AOK30 isolated from young plants of mountain laurel had a broad and intense anti microbial spectrum against various yeasts and fungal pathogens of Ericaceae. Taechowisan and Lumyong (2003) noted that metabolites of endophytic

actinomycetes from root tissues of *Zingiber officinale* and *Alpinia galanga* have extensive inhibition to the growth of phytopathogenic fungi such as *Colletotrichum musae* and *Fusarium oxysporum* f.sp. *cubense* with 35.7 mm and 28.4 mm as the diameter of growth inhibition zone respectively. Cao *et al.* (2004b) noted that of the endophytic *Streptomyces* strains isolated from surface sterilized tomato roots, 21% isolates produced antibacterial metabolites and 41% produced antifungal metabolites. 32% of isolates produced metabolites active against *Rhizoctonia solani* causing damping off disease in tomato seedlings. Tan *et al.* (2006) reported that the endophytic actinomycete isolate with antibacterial activity against *R.solanacearum* under *invitro* conditions was higher in healthy plants than from wilted plants. It was considered that antibacterial metabolites produced by antagonists were involved in protecting plants from the pathogen.

Min *et al.* (2007) reported that 26 endophytic actinomycetes had antagonistic action against the pathogens, *Botrytis cineria* and *Alternaria solani* in tomato. In green house experiments, strain BARI-5 was the most effective strain against tomato leaf mould. Ying *et al.* (2007) observed the efficacy of 50 strains of endophytic actinomycetes against Zucchini powdery mildew disease under green house experiments. The application of cell free culture filtrates of strains GKSHJA and PRI-8 showed 60.98% and 63.22% disease control efficiency respectively. In the field experiment, the disease control efficiency of the two strains were 56.33% and 69.88% respectively after the application of cell free filtrates diluted five times to naturally infected plants.

According to Xuan *et al.* (2008), out of the 77 strains of endophytic actinomycetes isolated from roots, stem and leaves of cucumber seedlings, 46.8% showed antifungal activities and 39% showed antibacterial activity. Lee *et al.* (2008) conducted a study to select endophytic actinomycetes as biocontrol agents against Chinese cabbage club root disease caused by *Plasmodiophora brassicae.* The genera *Microbiospora, Streptomyces* and *Micromonospora* when inoculated in germinated cabbage seeds and then transplanted to pots effectively suppressed

the occurrence of a post inoculated strain of *Plasmodiophora brassicae* in pots. de-Oliveira (2010) reported that *S.pluricolorescens*, an endophytic actinomycete isolated from tomato plants in Brazil showed 86.6% antimicrobial activity against at least one pathogen in tomato.

2.9. EFFECT OF ENDOPHYTIC ACTINOMYCETES ON PLANT GROWTH PROMOTION

As plant growth promoters, endophytes enhance growth including the formation of increased number of lateral roots and root hairs (Pillay and Nowak, 1997) in addition to an increase in plant height, shoot weight and shoot diameter (Yates *et al.,* 1997). Moura and da-Romeiro (2000) reported the antagonistic activity of 26 actinomycetes against *R.solanacearum*. The seedlings were planted in pots with artificially infested soil in a green house. Out of the 26 isolates, six were best plant growth promoting ones. Efficiency of a given isolate reflected increase in growth of all parameters used for evaluation except the time for seed germination. Actinomycetes with a positive growth promotion effect were also effective for biological control of bacterial wilt of tomato. Principal component analysis indicated that the variables such as leaf area, number of leaves and plant height at 20, 18 and 4 days after transplanting were the ones that best detected as treatment changes.

Igarashi *et al.* (2002b) observed pteridic acid A and B from the fermentation broth of *Streptomyces hygroscopicus* TP-A045 isolated from *Pteridium aquilinum* as plant growth promoters with auxin like activity. These compounds accelerated formation of adventitious roots in hypocotyls of kidney beans as effective as indole acetic acid. Igarashi *et al.* (2005) also investigated the effect of endophytic actinomycetes in plant growth promotion. Crop seeds were inoculated with the spores of endophytic actinomycetes and were grown in a green house. Of the tested microbes, *Streptomyces hygroscopicus* S-17 induced significant growth promotion of tomato which was two times in height and eight times in fresh

weight compared to the control. Meugro *et al.* (2006) reported that a strain of *Streptomyces* sp. MBR-52 accelerated the emergence and elongation of adventitious roots in plants. When tissue cultured seedlings of rhododendron were treated with MBR-52 in flasks, prominently accelerated the emergence of adventitious roots suggesting that this strain might have released some rooting plant hormones. Franco *et al.* (2006) noted that the application of endophytic actinomycetes as seed inoculants has proven to be beneficial for cereal crops as they influence plant growth through enhanced germination. El-Tarabily (2008) reported that the ability of *Streptomyces filipinensis* to promote the growth of tomato through the production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase under green house conditions. Among the various isolates, *S.filipinensis* induced largest increase in root and shoot length (62% and 54% respectively) and also in shoot dry weight (69%) when compared to non inoculated plants. Its ability to enhance plant growth was through the reduction in *in planta* levels of endogenous ACC and the consequent lowering of endogenous ethylene levels in plant tissues.

Hasegawa *et al.* (2008) evaluated the root growth accelerating activity of *Streptomyces* sp. MBR-52 from tissue cultured seedlings of rhododendron. The dry weight of adventitious roots increased in response to treatment with an aqueous solution of 0.05 mg/ml of metabolites from MBR-52. El-Tarabily *et al.* (2009) evaluated the potential of three endophytic actinomycetes namely *Actinoplanes companulatus, Micromonospora chalcea* and *Streptomyces spiralis* in the plant growth promoting activity of cucumber. All the three endophytic actinomycetes significantly increased root and shoot production and they were able to produce maximum level of growth promotion when they were combined together. The growth promotion effects of isolates appeared to have resulted in the enhanced root and shoot production through the activity of indole-3-acetic acid (IAA), indole-3-pyruvic acid (IPyA), Gibberlic acid (GA) and isopentenyl adenine (iPa).

2.8.1. Plant growth promotion through production of indole-3-acetic acid (IAA) by endophytic actinomycetes

Production of IAA by endophytic actinomycetes have been reported previously by many workers (Hirata, 1959; El-Sayed *et al.,* 1987; El-Shanshoury, 1991). Khamna *et al.* (2009) isolated actinomycetes from 16 medicinal plants and tested for the production of IAA, which showed a range of 5.5-144 µg/ml. Of these isolates *Streptomyces* sp. CMU-H009 isolated from lemon grass showed the highest ability to produce IAA at the rate of 143.95 µg/ml. de-Oliveira *et al.* (2010) reported that 72.1% of endophytic actinomycetes isolated from tomato plants in Southern Brazil showed a positive reaction for IAA production. Nimnoi *et al.* (2010) noted that isolates of endophytic actinomycetes from healthy shoots and roots of *Aquilaria crassna* (eagle wood) produced IAA in the range of 9.85 to 15.14 µg/ml.

Suttiviriya *et al.* (2008) reported that endophytic actinomycetes strains isolated from Thai plants produced IAA ranging from 0.17µg/ml to18.62 µg/ml. Solans *et al.* (2011) evaluated IAA production by root associated actinomycetes isolated from the actinorhizal plant *Ochetophila trinervis*. IAA production was eight times higher for *Micromonospora* sp. (9.03 ng/ml) followed by *Actinoplanes* sp. (0.27 ng/ml) and *Streptomyces* sp. (0.75 ng/ml).

Materials and Methods

3. MATERIALS AND METHODS

A study on 'Bioeffficacy of endophytic actinomycetes on plant growth promotion and management of bacterial wilt in tomato' was carried out in the Department of Plant Pathology, College of Horticulture, Vellanikkara during the year 2009-11. Details of materials used and the methods followed for the study are presented below.

3.1. ISOLATION OF ENDOPHYTIC ACTINOMYCETES

Endophytic actinomycetes were isolated from healthy tomato plants collected from high wilt incidence areas of Vellanikkara, Cherumkuzhy, Elanad (Thrissur district) and low wilt incidence areas of Ozhalapathy, Eruthenpathy (Palakkad district) (Dinakar *et al.,* 2005). Both young (less than two weeks) and old plants (more than two weeks) were obtained from different locations of above mentioned areas. The whole plant was uprooted manually and brought to the laboratory. The samples were washed under running tap water. The root and stem segments of 2-3 cm in length were made using a sterilized scalpel. For younger plants, root segments were taken just below the soil line and stem segments were taken 1-2 cm above the soil line. For older plants, root segments were taken 5-10 cm below the soil line and stem segments were taken 10 cm above the soil line.

The outer skin of the plant samples were gently scraped off to remove the external contamination. The stem and root segments of one gram (g) were weighed separately and surface sterilized with 70% ethanol for 30 seconds, followed by 1.05% sodium hypochlorite (five minutes) for root samples and 20% hydrogen peroxide (10 minutes) for stem samples. The samples were then soaked in 10% sodium bicarbonate solution for 10 minutes to disrupt the growth of endophytic fungi. The samples were then rinsed four times in 0.02 M sterile phosphate buffer of pH 7. An aliquot of 0.1 ml from the final buffer wash was

spread over Kenknights agar media (KAM) (Appendix I) which served as a sterility check. Each plant sample was ground in 9.9 ml of the final buffer wash using a sterile pestle and mortar. From this, 0.1 ml of the triturate was taken and spread over solidified KAM in sterile Petri dishes using a sterilized glass spreader. The plates were incubated at 28^0C for two weeks. The growth of endophytic actinomycetes was observed from fourth day onwards. Single isolated actinomycete colonies were transferred to KAM slants and were maintained as pure cultures by subculturing for further studies.

3.2. ISOLATION OF BACTERIAL WILT PATHOGEN

The pathogen causing bacterial wilt in tomato was isolated from the naturally infected plants (Kelman, 1954). The infected plant samples were uprooted and washed in running tap water to remove the adhering soil particles. The samples were then air dried and was subjected to ooze test. After confirmation of bacterial wilt disease, the stem portion of infected plants was cut into small pieces and surface sterilized with 1% sodium hypochlorite for five minutes followed by rinsing in three changes of sterile water. The sample bits were then gently crushed on a sterilized glass slide with a drop of sterilie water and the suspension was streaked on Triphenyl Tetrazolium Chloride (TZC) medium (Appendix I) in a Petri dish.

The plates were incubated at room temperature for 48 h. Typical colonies of bacterial wilt pathogen were selected, purified by repeated streaking and sub cultured on Nutrient agar (NA) (Appendix I) slants. The culture was preserved by taking few loopful of bacteria from single colonies on agar plate and suspended in sterile water in eppendorf tubes and stored at both room temperature and refrigerated conditions for further use as a stock culture to prevent the loss of virulence. The pathogenicity of the bacterial isolate was confirmed by the root dipped inoculation method (Winstead and Kelman, 1952) on the 20 day old tomato seedlings. The roots of the seedling were dipped in 10^7 cfu/ml

concentration of *R. solanacearum* suspension. Inoculated seedlings were transplanted in 10 cm pots filled with sterilized soil and placed at 30^0 C in green house. Symptom developments were observed at 14 days after inoculation.

3.3. *In vitro* EVALUATION OF ANTAGONISTIC EFFECT OF ENDOPHYTIC ACTINOMYCETES ON *Ralstonia solanacearum*

Antagonistic effect of five endophytic actinomycetes on bacterial wilt pathogen was evaluated by dual culture method (Tan *et al.*, 2006). For this bacterial suspension was prepared at 10^{-3} dilution and 0.1 ml from this was spread over solidified Potato Dextrose Agar (PDA) medium (Appendix I) in Petri plates using a sterile glass spreader. A five mm disc of agar block containing one week old growth of the antagonist was placed at the centre of the bacterial lawn. Plates containing monoculture of the pathogen maintained served as control. The design of the experiment was CRD. Three replications were maintained for each isolate. The plates were incubated at room temperature and observed for the antagonistic activity. Inhibition zone developed was measured and per cent inhibition was calculated as suggested by Vincent (1927).

$$PI = \frac{C - T}{C} \times 100$$ where

PI = Per cent Inhibition

C = Growth of the pathogen in control plates (cm)

T = Growth of the pathogen in dual culture (cm)

3.4. EFFECT OF CULTURE FILTRATE OF ENDOPHYTIC ACTINOMYCETES AGAINST *R. solanacearum*

One ml suspension of each isolate at 10^{-1} dilution was added into 25 ml sterile Kenknights broth taken in conical flasks. The flasks were kept undisturbed

for 20 days so as to obtain the secondary metabolites from the isolates. After 20 days of incubation, the cell free culture filtrate of the isolates was prepared using a millipore filter of size 0.2µ. From the culture filtrate, 0.1 ml was poured into a well of eight mm diameter made at the centre of the bacterial lawn. Plates with 0.1 ml sterile water poured into the well at centre of bacterial lawn, served as the control. The design of the experiment was CRD. Three replications were kept for each isolate. The plates were incubated at room temperature and observed for antagonistic activity. Inhibition zone was measured and per cent inhibition was calculated using the formula as given in 3.3.

3.5. IDENTIFICATION OF ENDOPHYTIC ACTINOMYCETES

The endophytic actinomycetes isolates were identified based on their morphological and cultural characters.

3.5.1. Morphological and cultural characters

Morphological characters such as presence or absence of substrate and aerial mycelium, fragmentation and sporulation of substrate mycelium, sclerotia formation and aerial mycelium with chains of arthrospores, spore chain morphology, spore surface ornamentation and motility of spores were studied by light microscopy (Shimizu *et al.,* 2000). Cultural characters such as colony size, color of spore mass, pigmentation of substrate mycelium and diffusible pigment production were studied based on standard keys. (Locci, 1989)

3.5.1.1. Color of spore mass

Spore masses were compared against the seven color series of blue, gray, green, red, violet, white and yellow (Pridham and Tresner, 1974b).

3.5.1.2. Pigmentation of substrate mycelium (colony reverse)

Colors were allotted to one of the five categories namely yellow- brown (which included strains lacking distinctive pigmentation), red- orange, green, blue or violet.

3.5.1.3. Diffusible pigment production

The colors were allotted to the same five categories which were used for substrate mycelium pigmentation.

3.6. QUANTITATIVE ESTIMATION OF INDOLE ACETIC ACID (IAA) PRODUCTION BY ENDOPHYTIC ACTINOMYCETES

The production of IAA by endophytic actinomycetes was determined according to the method of Bano and Mussarat (2003). One ml suspension of each isolate (at 10^{-1} dilution) grown on Yeast extract- Malt extract (YM) agar medium (Appendix I) and incubated at 30^0 C for five days, was added to five ml YM broth containing 2 mg/ml L-Tryptophan. These cultures were 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 (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) and incubated at 28^0 C for 30 min. The appearance of a pink color indicated IAA production. Optical density (OD) was read at 530 nm. The level of IAA produced was estimated against the IAA standards.

3.7. STUDIES ON MECHANISM OF ACTION OF ENDOPHYTIC ACTINOMYCETES

3.7.1. Production of siderophores by endophytic actinomycetes

The five endophytic actinomycetes isolates were tested for the iron dependent production of siderophores following the standard protocol of Kloepper

et al.(1980). 10 ml YM broth was amended with different concentrations of iron (0 ppm, 1 ppm and 10 ppm $FeCl_3$. $6H_2O$). Then, it was inoculated with one ml culture suspension of isolates separately and incubated for seven days at 28^0 C in a slow shaking condition. Later, the cells were pelleted by centrifugation at 10,000 rpm for 10 min and the supernatant was collected. The concentration of siderophores in the supernatant was read at 420 nm.

3.7.2. Production of hydrogen cyanide by endophytic actinomycetes

The isolates were evaluated for HCN production following the method of Wei *et al.* (1991). 0.5 ml culture suspension of isolates was inoculated into 10 ml nutrient broth supplemented with 4.4 g/l of Glycine taken in sterile Petri plates. Sterile filter paper discs soaked in picric acid solution (2.5 g picric acid + 12.5 g sodium carbonate in 1000 ml water) were placed in the lid of each plate. The Petri dishes were sealed with parafilm and incubated for five days. Change in the color of discs from yellow to brown and to red indicated the production of HCN.

3.7.3. Production of ammonia by endophytic actinomycetes

Ammonia production by the isolates was qualitatively estimated following the method of Nimnoi *et al.* (2010). One ml culture of isolates was inoculated into 25 ml of peptone water (Appendix I) and incubated at 30^0 C with shaking at 120 rpm for three weeks. After incubation 0.5 ml of Nessler's reagent was added to 10 ml broth. Development of brown to yellow color was recorded as positive for ammonia production.

3.7.4. Production of non-volatile metabolites by endophytic actinomycetes

The production of non- volatile metabolites by the isolates was determined following the method of Dennis and Webster (1971b). A sterile cellophane disc was placed over solidified PDA medium in Petri plates. Then 0.1 ml suspension

of isolate was spread over the sheet using a glass spreader. The plates were incubated at 28^0 C for five days. Then the cellophane disc along with the adhering antagonist was carefully removed using a forceps. A loopful of pathogen was then streaked over the medium and inhibition in its growth was noticed.

3.8. EVALUATION OF ENDOPHYTIC ACTINOMYCETES AGAINST BACTERIAL WILT PATHOGEN UNDER POT CULTURE CONDITIONS

A pot culture experiment was laid to find out the efficacy of endophytic actinomycetes isolates on plant growth promotion and management of bacterial wilt in tomato. The experiment was conducted during October-January 2010, at College of Horticulture, Vellanikkara. The details of experiment are as follows:

Design - CRD
Treatments - 14
Replications - 3
Variety - Pusa Ruby
No. of plants/treatment - 9

The treatment details for the experiment were as follows:

T_1 – EAVK
T_2 – EACK
T_3 – EAEN
T_4 – EAOP
T_5 – EAET
T_6 – Urea (@44 g m^{-2}) + Lime (@ 500 g m^{-2})
T_7 – Copper hydroxide @ 0.2% (soil drenching)
T_8 – Control (with pathogen alone)

Antagonists (10^4 spores/ml) were applied at the time of planting, 45 DAP and 60 DAP as soil drenching @ 30 ml/plant. The time of application of urea + lime and copper hydroxide was same as that of the antagonists. Challenge inoculation of pathogen (10^5 cfu/ml) was done at 30 DAP using fresh bacterial suspension @ 10 ml/plant.

Treatments T_9–T_{14} were maintained for taking biometric observations alone, without the challenge inoculation of the pathogen.

T_9 – EAVK

T_{10} – EACK

T_{11} – EAEN

T_{12} – EAOP

T_{13} – EAET

T_{14} – Absolute control (without pathogen and antagonists)

3.8.1. Preparation of potting mixture and planting

The potting mixture consisting of sand: soil: cowdung @ 1:1:1 was prepared. It was sterilized by drenching 5% formaldehyde solution and was covered with a polythene sheet for 14 days. Later, it was kept open for a period of 7-10 days with occasional turning, to remove any traces of formaldehyde. Sterilized potting mixture was filled in earthen pots of size 12″ x 12″. Tomato variety Pusa Ruby was used for the study. Seeds were sown in the nursery and one month old seedlings were transplanted in pots.

3.8.2. Observations recorded

Observations on per cent wilt incidence, per cent wilt index, plant height, days to flowering, days to first harvest, number of fruits per plant, per fruit weight and yield per plant were recorded.

3.8.2.1. Per cent wilt incidence

The incidence of wilt was calculated using the formula:

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

3.8.2.2. Per cent wilt index

Per cent wilt index was calculated as suggested by Winstead and Kelman (1952). Disease readings were usually made at weekly intervals by classifying each plant according to one of the following numerical grades: no symptoms, 0; one leaf partially wilted, 1; two or three leaves wilted, 2; all except top two or three leaves wilted, 3; all leaves wilted, 4; and dead, 5. The number of plants in each symptom category was multiplied by the corresponding numerical grade and the products were added. The summation was converted to a disease index value by dividing by the maximum numerical grade for the given number of plants and multiplying by 100.

3.8.2.3. Plant height

The distance from the base of the plant to the tip was taken as height at 15 days interval and expressed in centimeters.

3.8.2.4. Days to flowering

Number of days taken from transplanting to opening of first flower was recorded and mean was found out.

3.8.2.5. Days to first harvest

Number of days taken from transplanting till the fruit color turned yellow was recorded and mean was found out.

3.8.2.6. Number of fruits per plant

Total numbers of fruits were counted from each plant and mean was worked out.

3.8.2.7. Per fruit weight

Mean weight of each fruit was recorded and expressed in grams.

3.8.2.8. Yield per plant

Mean weight of fruit was multiplied with the total number of fruits per plant and expressed in gram.

3.9. 16S rRNA SEQUENCE ANALYSIS OF SELECTED ENDOPHYTIC ACTINOMYCETES ISOLATES

After the evaluation of endophytic actinomycetes against bacterial wilt pathogen under pot culture experiment, best three isolates (EACK, EAOP and EAEN) were selected and 16S rRNA sequence analysis was carried out. A rapid method for identifying filamentous actinomycetes genera was used based on the amplification of 16S rRNA gene using Polymerase Chain Reaction (PCR) (Cook and Meyers, 2003).

3.9.1. Amplification of 16S rRNA gene

A single colony was taken by inoculation loop, mixed with 10 µl sterile water and kept at 94^0C for two minutes. After a brief centrifugation to sediment the actinomycete cell constituents, 1 µl of supernatant was taken and used as a template for amplification of 16S rRNA gene. The details of the primer used are given below.

Primer details	Sequence 5' – 3'	Base pair
27 f	AGAGTTTGATCCTGGCTCAG	20
1492 r	TACGGYTACCTTGTTACGACTT	22

Polymerase chain reaction was carried out in Eppendorf Master Cycler (Gradient, Germany). The composition of the reaction mixture for PCR is as follows:

Component	Per reaction volume required
Template	1.0 µl
10X Taq buffer A	2.5 µl
d NTP mix (10 mM)	1.0 µl
Forward primer	1.0 µl
Reverse primer	1.0 µl
Taq DNA polymerase (0.3 U)	2.0 µl
Distilled water	16.5 µl

Total	25 µl

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

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

3.9.2. Agarose gel electrophoresis

The quality of isolated DNA was evaluated through agarose gel electrophoresis (Sambrook *et al.,* 1989). 1X TAE buffer was prepared from the 50 X TAE (pH 8.0) stock solutions. Agarose (Genei, Low EEO) (0.9 per cent) was weighed and dissolved in TAE buffer by boiling. Ethidium bromide prepared from a stock of 10 mg ml^{-1} was added to it at a concentration of 0.5 $\mu g ml^{-1}$ and mixed well. The open end of gel casting tray was sealed with cellotape, the comb was placed properly and dissolved agarose was poured into the tray. The gel was placed in the electrophoresis unit after 30 min with the well side directed towards the cathode. 1X TAE buffer was added to the buffer tank (Genei, Bangalore) so as to cover the well with a few mm of buffer. 5 µl DNA sample was mixed with 1 µl tracking dye (6X) and carefully loaded into the wells using a micropipette. The λ DNA/ *Eco* RI/ *Hind* III Double Digest (Genei, Bangalore) were used as the

molecular weight marker. The cathode and the anode of the electrophoresis unit were connected to the power pack (Hoefer, USA) and the gel was run at constant voltage of 100 V. The power was turned off when the tracking dye reached at about 3 cm from the anode end.

3.9.3. Gel documentation

The DNA bands separated by electrophoresis were viewed and photographed using Vision Works LS software and UVP GelDoc- IT™ imaging system.

3.9.4. Direct purification of PCR product

20 µl of the PCR product was purified using PCR purification kit (Bioserve) following the procedure as per the manufactures guidelines.

3.9.5. Sequencing of the purified product

The purified product was sequenced at Seigenome Pvt. Ltd. Cochin using the primers 27f and 1492r.

3.9.6. Nucleotide sequence analysis

The Blastn programme (http://www.ncbi.nlm.nih.gov/blast/) was used to find out the homology of the nucleotide sequences.

3.10. STATISTICAL ANALYSIS

Analysis of variance was done on the data collected using the statistical package MSTAT (Freed, 1986). Multiple comparisons among the treatment means were done using DMRT.

Results

4. RESULTS

The experimental results obtained from the studies on 'Bioeffficacy of endophytic actinomycetes on plant growth promotion and management of bacterial wilt in tomato' are presented below.

4.1. ISOLATION OF ENDOPHYTIC ACTINOMYCETES

Endophytic actinomycetes were isolated from the stem and roots of healthy tomato plants collected from five different locations *viz;* Vellanikkara, Cherumkuzhy, Elanad, Ozhalapathy and Eruthenpathy (Table 1 and Plate 1). The isolates of endophytic actinomycetes obtained from tomato plants were designated as EAVK (Vellanikkara), EACK (Cherumkuzhy), EAEN (Elanad), EAOP (Ozhalapathy) and EAET (Eruthenpathy). Only a single type of actinomycete isolate was obtained from each location. Among the isolates, EACK, EAOP and EAET were obtained from the roots of the sample whereas EAVK and EAEN were obtained from the stem. The maximum count was obtained from the sample collected from Cherumkuzhy (EACK) ($8x10^1$ cfu/g of sample), followed by Eruthenpathy (EAET) ($3x10^1$ cfu/g of sample). The minimum count of endophytic actinomycetes was obtained from Vellanikkara (EAVK), Elanad (EAEN) and Ozhalapathy (EAOP) ($1 x10^1$ cfu/g of sample). The stem of samples recorded less number of isolates when compared to the roots.

4.2. ISOLATION OF BACTERIAL WILT PATHOGEN

The pathogen was isolated from wilted tomato plants on TZC agar medium (Plate 2). Isolation of bacterium on TZC medium yielded circular, smooth, convex, fluidal, slimy and creamy white colonies with light pink centre, after 48 h of incubation indicating its identity as *R. solanacearum* (Plate 3). The purified

Table 1. Enumeration of endophytic actinomycetes isolated from high and low bacterial wilt incidence areas of tomato

Sl. No.	Isolates	Location	District	Tomato variety	Isolate obtained from	Microbial count (x 10^1 cfu/g sample)
1	EAVK	Vellanikkara	Thrissur	Anagha	Stem	1
2	EACK	Cherumkuzhy	Thrissur	Anagha	Root	8
3	EAEN	Elanad	Thrisur	Anagha	Stem	1
4	EAOP	Ozhalapathy	Palakkad	Local	Root	1
5	EAET	Eruthenpathy	Palakkad	Super Lekshmi	Root	3

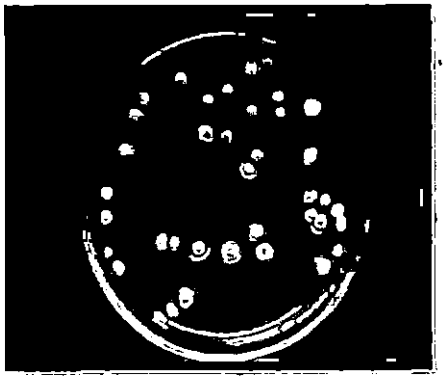
EAVK- Vellanikkara isolate

EACK -Cherumkuzhy isolate

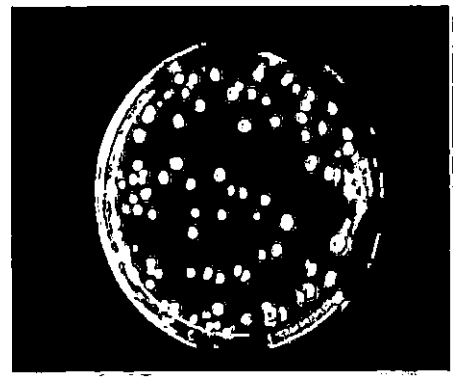
EAEN - Elanad isolate

EAOP- Ozhalapathy isolate

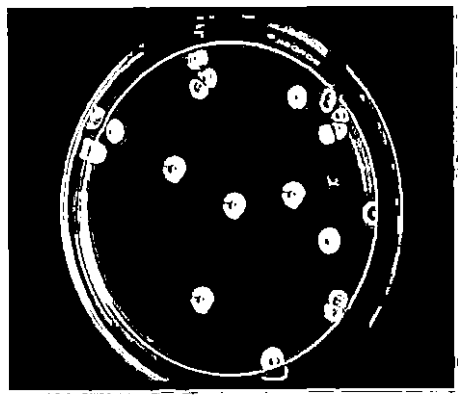
EAET- Eruthenpathy isolate



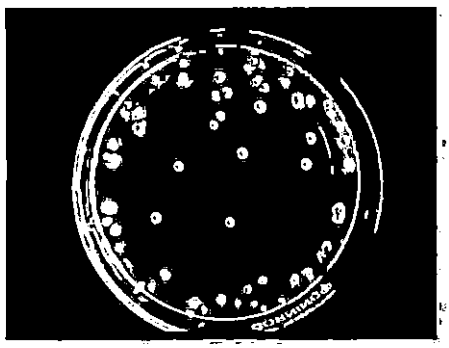
EAVK (Vellanikkara)



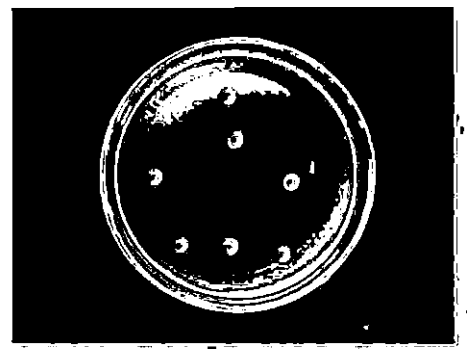
EACK (Cherumkuzhy)



EAEN (Elanad)



EAOP (Ozhalapathy)



EAET (Eruthenpathy)

Plate 1. Endophytic actinomycetes obtained from tomato plants

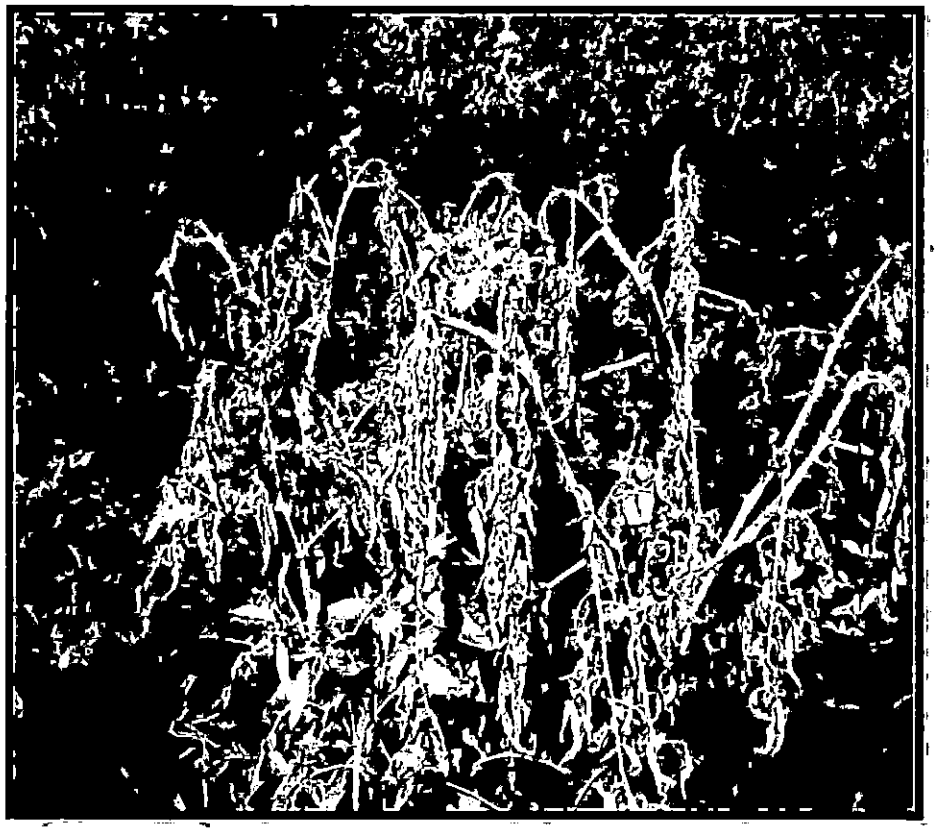


Plate 2. Symptoms of bacterial wilt incidence in tomato

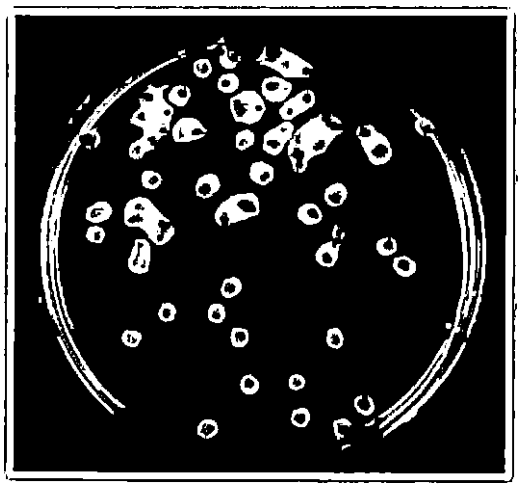


Plate 3. Typical colonies of *Ralstonia solanacearum* in TZC medium

culture was preserved in slants as well as in sterile water and stored under refrigerated conditions for further studies. Pathogenicity of the organism was established by re-isolating colonies resembling the original isolate of the bacterium from the inoculated seedlings.

4.3. *In vitro* EVALUATION OF ANTAGONISTIC EFFECT OF ENDOPHYTIC ACTINOMYCETES ON *Ralstonia solanacearum*

The efficacy of endophytic actinomycetes isolates against *R.solanacearum* was evaluated under *in vitro* conditions (Table 2). Out of the five isolates, maximum inhibition (29.25%) of pathogen was recorded with the Vellanikkara isolate (EAVK) (Plate 4a) which was on par with Ozhalapathy (EAOP) (22.59%) (Plate 4b) and Elanad isolates (EAEN) (Plate 4c) (20.00%).

4.4. EFFECT OF CULTURE FILTRATE OF ENDOPHYTIC ACTINOMYCETES AGAINST *R. solanacearum*

The efficacy of culture filtrate of endophytic actinomycetes against *R.solanacearum* was evaluated under *in vitro* conditions (Table 3). Among the isolates maximum inhibition (44.63%) of the pathogen was observed with the Cherumkuzhy isolate (EACK) which was on par with the Ozhalapathy isolate (EAOP) (42.59%) (Plate 5).

4.5. IDENTIFICATION OF ENDOPHYTIC ACTINOMYCETES BASED ON THEIR MORPHOLOGICAL AND CULTURAL CHARACTERS

The morphological and cultural characters of the endophytic actinomycetes were studied based on standard keys and the results are presented in Table 4.

Table 2. *In vitro* evaluation of antagonistic effect of endophytic actinomycetes against *Ralstonia solanacearum*

Sl. No.	Isolate	Per cent inhibition
1	EAVK	29.25^{a}
2	EACK	12.59^{bc}
3	EAEN	20.00^{ab}
4	EAOP	22.59^{ab}
5	EAET	8.14^{c}
6	Control	0.00
	CD (0.05)	9.6

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

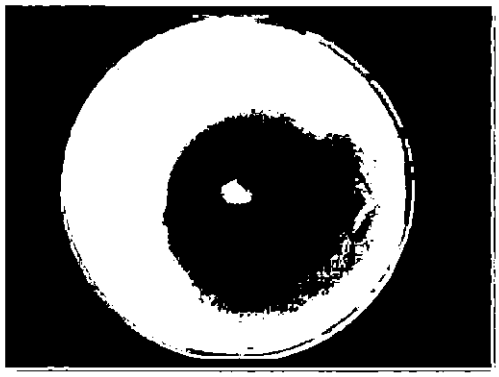
EAVK- Vellanikkara isolate

EACK -Cherumkuzhy isolate

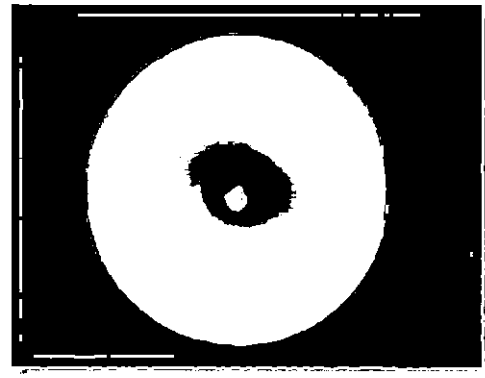
EAEN - Elanad isolate

EAOP- Ozhalapathy isolate

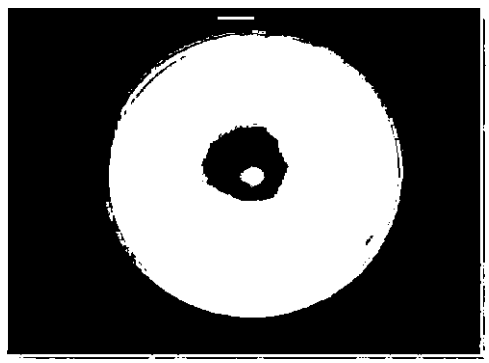
EAET- Eruthenpathy isolate



a. EAVK (Vellanikkara)



b. EAOP (Ozhalapathy)



c. EAEN (Elanad)

Plate 4. Effect of endophytic actinomycetes against *Ralstonia solanacearum* under *in vitro* conditions

Table 3. Effect of culture filtrate of endophytic actinomycetes against *R. solanacearum*

Sl. No.	Isolate	Per cent inhibition
1	EAVK	27.22[b]
2	EACK	44.63[a]
3	EAEN	26.11[b]
4	EAOP	42.59[a]
5	EAET	31.11[b]
6	Control	0.00
	CD (0.05)	6.1

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

EAVK- Vellanikkara isolate

EACK -Cherumkuzhy isolate

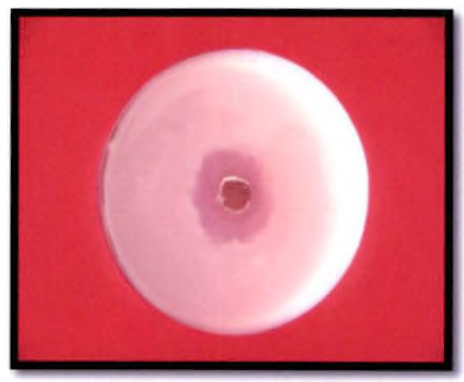
EAEN - Elanad isolate

EAOP- Ozhalapathy isolate

EAET- Eruthenpathy isolate

EAVK (Vellanikkara)

EACK (Cherumkuzhy)



EAEN (Elanad)

EAOP (Ozhalapathy)

EAET (Eruthenpathy)

Plate 5. Effect of culture filtrate of endophytic actinomycetes against *Ralstonia solanacearum* under *in vitro* conditions

4.5.1. Morphological and cultural characters

The colony size of all the isolates was medium. On the solid agar substratum, the branching network of hyphae developed by all the isolates grew both on the surface of the substratum and inside it forming a substrate mycelium. But chain of spores and sporangia were absent in the substrate mycelium of all the isolates. Fragmentation of substrate mycelium and motility of spores were absent for all the isolates. Later the colonies of all the isolates became covered with aerial mycelium bearing chain of arthrospores, which was initially white but assumed a range of colors when spore formation started. Color of the spore mass for all the isolates except Cherumkuzhy isolate was gray. Spiral spore chain morphology was observed for all the isolates and the spores were smooth surfaced. Red- orange colored, diffusible pigments were produced by Vellanikkara and Cherumkuzhy isolates which were absent for the other three isolates. The isolates from Vellanikkara and Cherumkuzhy produced red- orange colored pigmentation for the substrate mycelium whereas for the other three isolates distinctive pigmentation was absent. Based on these results the endophytic actinomycetes isolates obtained from tomato were tentatively identified to be genus *Streptomyces*.

4.6. QUANTITATIVE ESTIMATION OF INDOLE ACETIC ACID (IAA) PRODUCTION BY ENDOPHYTIC ACTINOMYCETES

A test was conducted to know the ability of endophytic actinomycetes in producing IAA (Plate 6). The appearance of pink color on adding Salkowski reagent to the supernatant indicated the level of IAA production, which was maximum for the Ozhalapathy isolate and least for the Vellanikkara isolate (Table 5). It was clear that among the isolates, EAOP produced the maximum

Table 4. Morphological and cultural characters of endophytic actinomycetes

Sl. No.	Character	Isolates				
		EAVK	EACK	EAEN	EAOP	EAET
1	*Colony size*	*Discrete*	*Discrete*	Discrete	Discrete	Discrete
2	Substrate mycelium	+	+	+	+	+
3	Chain of spores	-	-	-	-	-
4	Sporangia	-	-	-	-	-
5	Motile spores	-	-	-	-	-
6	Fragmentation of substrate mycelium	-	-	-	-	-
7	Aerial mycelium	+	+	+	+	+
8	Chain of arthrospores	+	+	+	+	+
9	Arthrospores in verticils	-	-	-	-	-
10	Spore chain morphology	Spirales	Spirales	Spirales	Spirales	Spirales
11	Spore surface ornamentation	Smooth	Smooth	Smooth	Smooth	Smooth
12	Color of spore mass	Gray	White	Gray	Gray	Gray
13	Pigmentation of substrate mycelium	Red-orange	Red-orange	-	-	-
14	*Diffusible* pigments	Red-orange	Red-orange	-	-	-
15	Probable genus	*Streptomyces*	*Streptomyces*	*Streptomyces*	*Streptomyces*	*Streptomyces*

+ presence - absence

Table 5. Production of indole acetic acid (IAA) by endophytic actinomycetes

Sl. No.	Isolates	Concentration of IAA (µg/ml)	Presence of IAA
1	EAVK	3.2	+
2	EACK	41.8	+++
3	EAEN	16.5	++
4	EAOP	73.1	++++
5	EAET	23.5	++

++++ Very high, +++ High, ++ Medium, + Low

EAVK- Vellanikkara isolate

EACK -Cherumkuzhy isolate

EAEN - Elanad isolate

EAOP- Ozhalapathy isolate

EAET- Eruthenpathy isolate

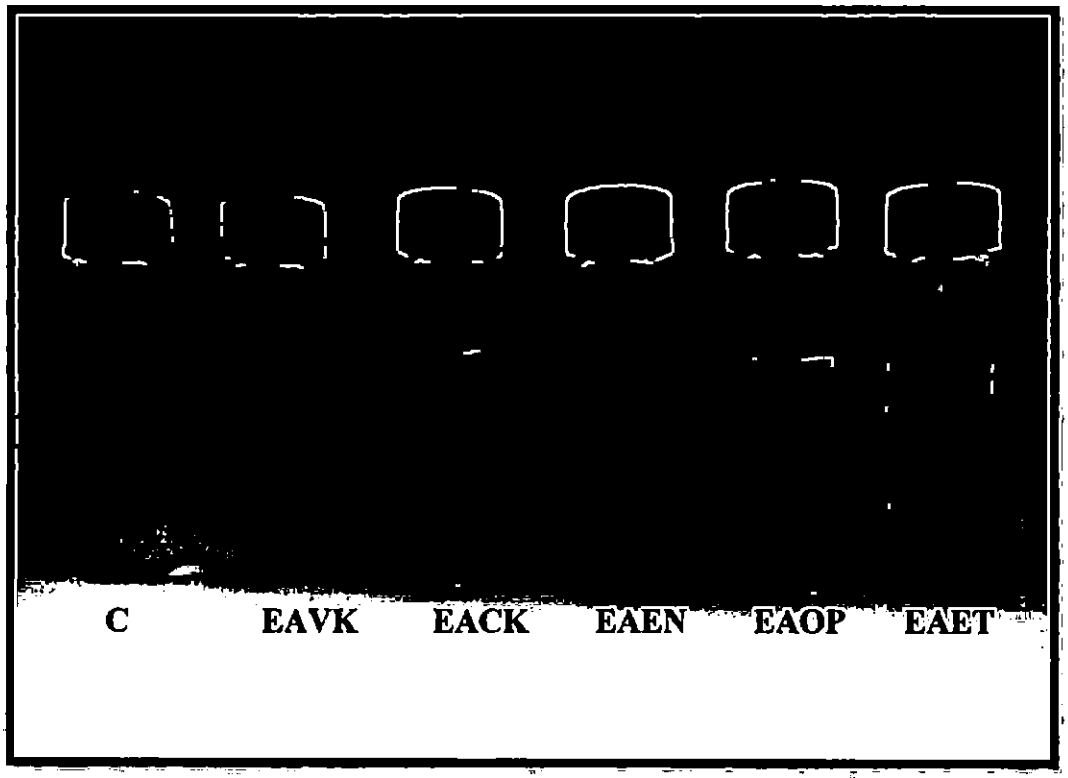


Plate 6. Production of IAA by endophytic actinomycetes

EAVK- Vellanikkara isolate

EACK -Cherumkuzhy isolate

EAEN - Elanad isolate

EAOP - Ozhalapathy isolate

EAET - Eruthenpathy isolate

concentration of IAA (73.1 µg/ml) followed by EACK (41.8 µg/ml) and the least was produced by EAVK (3.2 µg/ml).

4.7. STUDIES ON MECHANISM OF ACTION OF ENDOPHYTIC ACTINOMYCETES

4.7.1. Production of siderophores by endophytic actinomycetes

The ability of endophytic actinomycetes isolates to produce siderophores reduced as the concentration of ferric chloride increased in the broth (Table 6). Further, there was variation in the production of siderophores by different isolates (Figure 1). Without the addition of $FeCl_3$. $6H_2O$, the maximum siderophore was produced by EAOP isolate followed by EAVK isolate. At 1 ppm of $FeCl_3$. $6H_2O$, siderophores production of isolates decreased from the initial rate. The production further decreased at 10 ppm $FeCl_3$. $6H_2O$, where the least production was noticed for EAOP and EAVK isolates itself.

4.7.2. Production of hydrogen cyanide by endophytic actinomycetes

In vitro HCN production by endophytic actinomycetes isolates were tested following the method of picric acid assay. None of the isolates were found to be cyanogenic in nature as evidenced by no change in the color of filter paper from yellow to brown or to red.

4.7.3. Production of ammonia by endophytic actinomycetes

The production of ammonia by the isolates was detected by the change in the color of peptone water on addition of Nessler's reagent (Plate 7). The maximum production of ammonia was shown by EAVK, EACK and EAET

Table 6. Production of siderophores by endophytic actinomycetes at different concentrations of $FeCl_3.6H_2O$

Sl. No.	Isolates	OD value*		
		Concentrations of $FeCl_3.6H_2O$		
		0 ppm	1ppm	10 ppm
1	EAVK	0.826	0.587	0.579
2	EACK	0.602	0.592	0.584
3	EAEN	0.751	0.745	0.709
4	EAOP	0.841	0.671	0.511
5	EAET	0.730	0.693	0.671

EAVK- Vellanikkara isolate

EACK -Cherumkuzhy isolate

EAEN - Elanad isolate

EAOP- Ozhalapathy isolate

EAET- Eruthenpathy isolate

* Mean of three replications

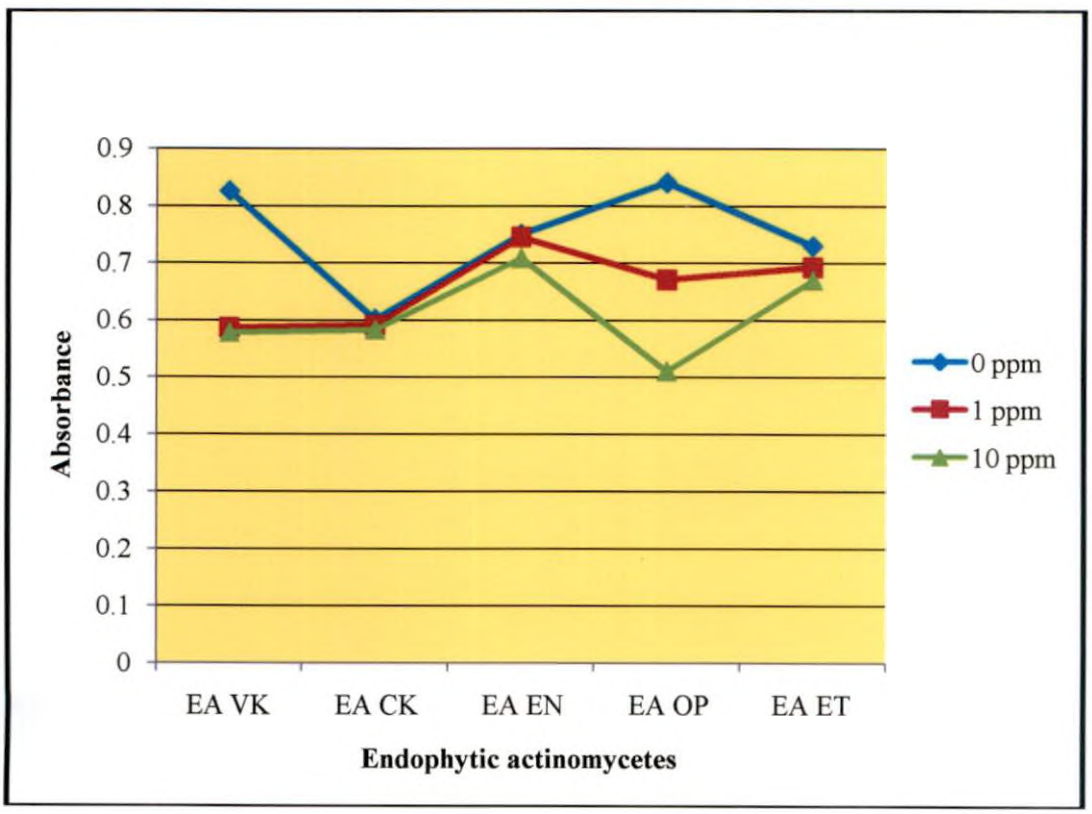


Fig.1. Production of siderophores by endophytic actinomycetes at various concentrations of ferric chloride

EAVK- Vellanikkara isolate

EACK -Cherumkuzhy isolate

EAEN - Elanad isolate

EAOP - Ozhalapathy isolate

EAET - Eruthenpathy isolate

Table 7. Production of ammonia by endophytic actinomycetes

Sl. No.	Isolates	Presence of ammonia
1	EAVK	+++
2	EACK	+++
3	EAEN	++
4	EAOP	+
5	EAET	+++

+++ High, ++ Medium, + Low

EAVK- Vellanikkara isolate

EACK -Cherumkuzhy isolate

EAEN - Elanad isolate

EAOP - Ozhalapathy isolate

EAET - Eruthenpathy isolate

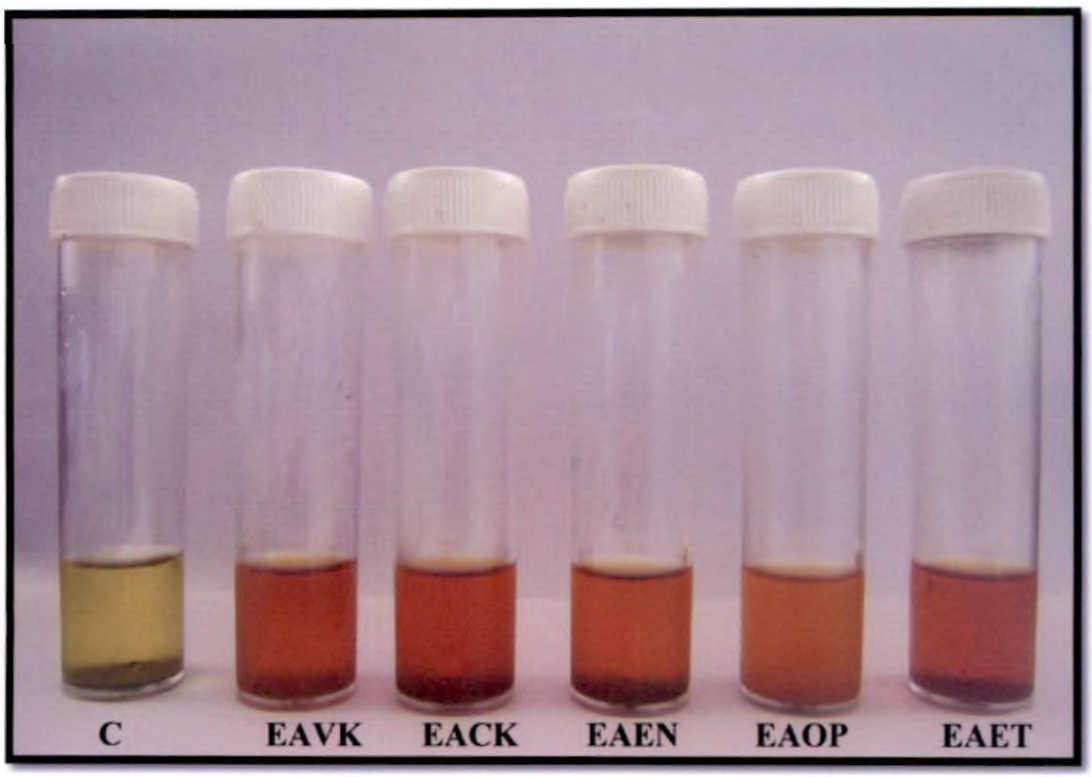


Plate 7. Production of ammonia by endophytic actinomycetes

EAVK- Vellanikkara isolate

EACK -Cherumkuzhy isolate

EAEN - Elanad isolate

EAOP - Ozhalapathy isolate

EAET - Eruthenpathy isolate

isolates as evidenced by the brown color and the least was produced by EAOP isolate (Table 7).

4.7.4. Production of non volatile metabolites by endophytic actinomycetes

All endophytic actinomycetes tested produced non volatile metabolites, which were confirmed from the complete inhibition of pathogen growth, when a loopful was streaked over the medium containing non volatile metabolites from the isolates.

4.8. EVALUATION OF ENDOPHYTIC ACTINOMYCETES AGAINST BACTERIAL WILT PATHOGEN UNDER POT CULTURE CONDITIONS

A pot culture experiment was conducted to evaluate the bio efficacy of endophytic actinomycetes on plant growth promotion and management of bacterial wilt in tomato (Plate 8). Parameters such as per cent wilt incidence, per cent wilt index, plant height, days to flowering, days to first harvest, number of fruits per plant, per fruit weight and yield per plant were recorded and the results are presented in Tables 8 to 12.

4.8.1. Per cent wilt incidence

Statistical analysis of data revealed that significant difference existed among various treatments. Minimum per cent wilt incidence (29.63%) was shown by plants treated with urea (44 g m^{-2}) and lime (500 g m^{-2}) (T_6) and differed significantly from the other treatments (Table 8). It was followed by the plants treated with EAOP (T_4) isolate (37.03%) which was on par with the plants treated with copper hydroxide (T_7) (40.74%). Maximum wilt incidence (87.96%) was noticed in plants inoculated with pathogen alone (Figure 2).

Plate 8. View of the pot culture experiment

Table 8. Effect of various treatments on bacterial wilt incidence

Treatment	Per cent wilt incidence
T_1 (EAVK)	$66.67(0.96)^{b}$
T_2 (EACK)	$55.56(0.84)^{bc}$
T_3 (EAEN)	$66.67(0.96)^{b}$
T_4 (EAOP)	$37.03(0.65)^{cd}$
T_5 (EAET)	$48.15((0.77)^{bcd}$
T_6 (Urea + lime)	$29.63(0.57)^{d}$
T_7 (Copper hydroxide)	$40.74(0.69)^{cd}$
T_8 (Control)	$87.96(1.24)^{a}$
CD (0.05)	16.6

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

Figures in parenthesis are arc- sine transformed values

EAVK - Vellanikkara isolate

EACK - Cherumkuzhy isolate

EAEN - Elanad isolate

EAOP - Ozhalapathy isolate

EAET - Eruthenpathy isolate

4.8.2. Per cent wilt index

Analysis of data on per cent wilt index revealed that significant differences existed among the treatments (Table 9). Minimum per cent wilt index (25.92 %) was shown by plants treated with urea (44 g m^{-2}) and lime (500 g m^{-2}) (T_6) and it differed significantly from the other treatments. It was followed by the plants treated with EAOP (T_4) isolate (36.30 %) which was on par with the plants treated with copper hydroxide (T_7) (38.51 %). Maximum wilt index (87.77 %) was noticed in plants inoculated with pathogen alone which differed significantly from all other treatments (Figure 2).

4.8.3. Plant height

The effect of endophytic actinomycetes on plant height was recorded at 15 days interval from the date of transplanting. There was no significant difference among the treatments with respect to plant height up to 45 DAP (Table 10). However, significant difference was observed among the treatments from 60 DAP. During this period, plants treated with EAOP isolate (T_{12}) recorded the maximum height (77.31 cm) which was on par with EAET (T_{13}) isolate (72.71 cm) and statistically superior to all other treatments. The lowest value in plant height (57.83 cm) was recorded by EACK (T_{10}) isolate. It was found to be statistically on par with T_{11} and T_{14} (Figure 3).

4.8.4. Days taken for flowering

Treatments differed significantly for days taken for the emergence of first flower (Table 11). Plants treated with EAEN (T_{11}) isolate took the minimum

Table 9. Effect of various treatments on per cent wilt index

Treatments	Per cent wilt index
T_1 (EAVK)	62.22^{b}
T_2 (EACK)	54.81^{bc}
T_3 (EAEN)	67.44^{b}
T_4 (EAOP)	36.30^{dc}
T_5 (EAET)	43.69^{cd}
T_6 (Urea + lime)	25.92^{c}
T_7 (Copper hydroxide)	38.51^{dc}
T_8 (Control)	87.77^{a}
CD (0.05)	16.6

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

EAVK - Vellanikkara isolate

EACK - Cherumkuzhy isolate

EAEN - Elanad isolate

EAOP - Ozhalapathy isolate

EAET - Eruthenpathy isolate

Table 10. Effect of different endophytic actinomycetes on plant height

Treatments	Plant height (cm)		
	30 DAP	45 DAP	60 DAP
T_9 (EAVK)	35.98[a]	56.57[a]	69.47[ab]
T_{10} (EACK)	38.67[a]	49.77[a]	57.83[b]
T_{11} (EAEN)	36.67[a]	55.78[a]	59.67[b]
T_{12} (EAOP)	40.26[a]	61.44[a]	77.31[a]
T_{13} (EAET)	37.42[a]	63.90[a]	72.71[a]
T_{14} (Control)	45.08[a]	50.33[a]	59.52[b]
CD (0.05)	11.2	13.0	11.1

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

Biometric observations were taken only for treatments numbered from T_9–T_{14}

DAP: Days after transplanting

EAVK - Vellanikkara isolate

EACK - Cherumkuzhy isolate

EAEN - Elanad isolate

EAOP - Ozhalapathy isolate

EAET - Eruthenpathy isolate

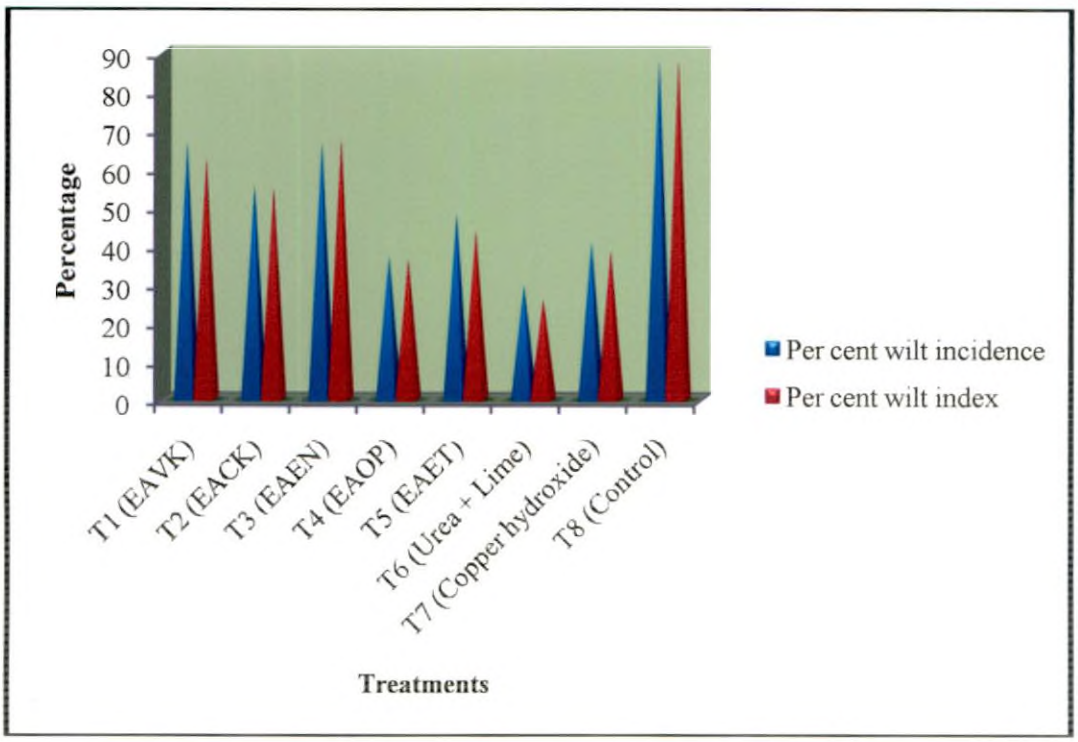


Fig.2. Effect of various treatments on per cent wilt incidence and per cent wilt index

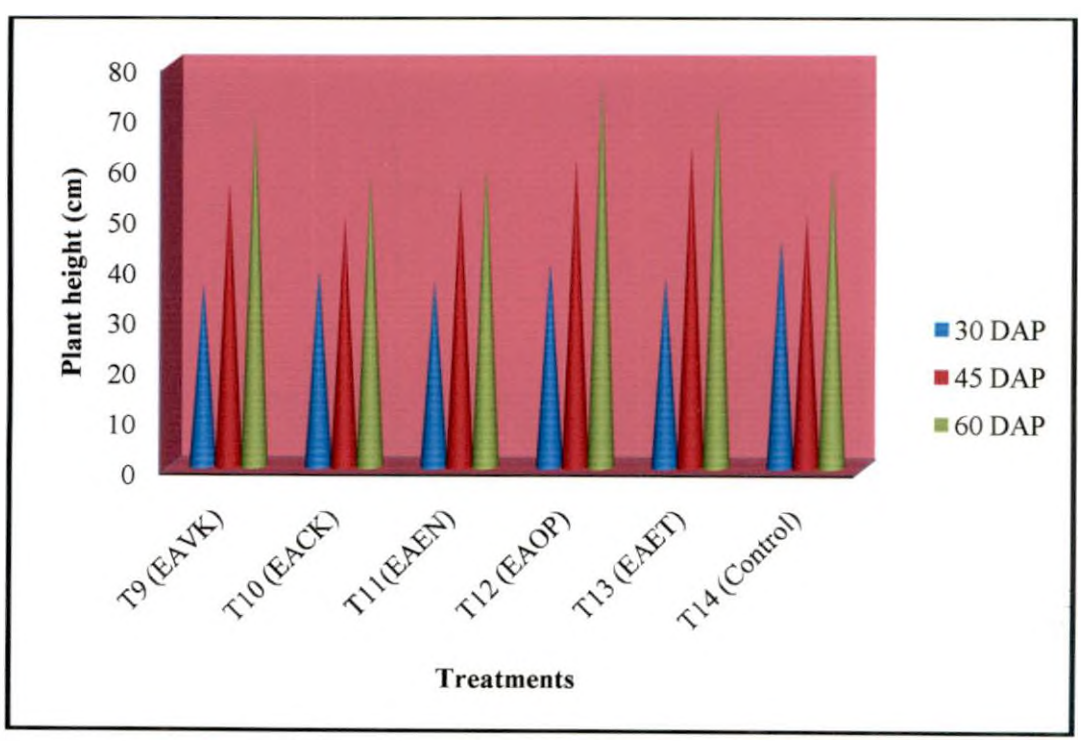


Fig.3. Effect of endophytic actinomycetes on plant height

Table 11. Effect of different endophytic actinomycetes on days to flowering

Treatments	Days to flowering (DAP)
T_9 (EAVK)	35.50^{ab}
T_{10} (EACK)	40.20^{ab}
T_{11} (EAEN)	34.83^{b}
T_{12} (EAOP)	39.39^{ab}
T_{13} (EAET)	36.77^{ab}
T_{14} (Control)	40.39^{a}
CD (0.05)	4.6

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

Biometric observations were taken only for treatments numbered from T_9–T_{14}

DAP: Days after transplanting

EAVK - Vellanikkara isolate

EACK - Cherumkuzhy isolate

EAEN - Elanad isolate

EAOP - Ozhalapathy isolate

EAET - Eruthenpathy isolate

number of days (34.83 DAP) for first flowering followed by EAVK (T_9) isolate (35.50 DAP) and were found to be statistically on par. Plants treated with EACK (T_{10}) isolate and the control (T_{14}) plants took the maximum number of days for flowering (40.20 and 40.39 DAP respectively) (Figure 4).

4.8.5. Days to first harvest

Days taken from fruit set to harvest as influenced by various treatments are presented in Table 12. The plants treated with EAVK (T_9) isolate took the minimum number of days (81.66 DAP) for the first harvest followed by EAET (T_{13}) isolate (82.45 DAP) and were found to be statistically on par. The control plants took the maximum number of days (86.38 DAP) for the first harvest (Figure 4).

4.8.6. Number of fruits per plant

The plants treated with EAET (T_{13}) isolate produced the maximum number of fruits per plant (17.57) followed by EAOP (T_{12}) isolate (16.25) and they were found to be statistically on par. The lowest number of fruits was observed in plants treated with EAVK (T_9) isolate (11.26) and the control plants (11.48) (Table 12 & Figure 5).

4.8.7. Per fruit weight

The data regarding per fruit weight indicated that the plants treated with EAOP (T_{12}) isolate produced fruits with maximum weight (20.30 g) which was on par with EAET (T_{13}) isolate (19.33 g) and was statistically superior to all other treatments (Plate 9). Per fruit weight was lowest for the control plants (11.96 g) (Table 12 and Figure 6).

Table 12. Effect of different endophytic actinomycetes on yield parameters of tomato plant

Treatments	Days to first harvest (DAP)	No. of fruits	Per fruit weight (g)	Fruit yield (g/plant)
T_9 (EAVK)	81.66^{b}	11.26^{b}	15.46^{bc}	161.65^{c}
T_{10} (EACK)	83.93^{ab}	13.75^{ab}	18.21^{ab}	226.65^{bc}
T_{11} (EAEN)	83.73^{ab}	13.57^{ab}	14.49^{cd}	182.67^{c}
T_{12} (EAOP)	85.01^{ab}	16.25^{ab}	20.30^{a}	316.81^{ab}
T_{13} (EAET)	82.45^{b}	17.57^{a}	19.33^{a}	332.02^{a}
T_{14} (Control)	86.38^{a}	11.48^{b}	11.96^{d}	131.75^{c}
CD (0.05)	3.0	4.3	9.0	85.5

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

Biometric observations were taken only for treatments numbered from T_9–T_{14}

DAP: Days after transplanting

EAVK- Vellanikkara isolate

EACK - Cherumkuzhy isolate

EAEN - Elanad isolate

EAOP - Ozhalapathy isolate

EAET - Eruthenpathy isolate

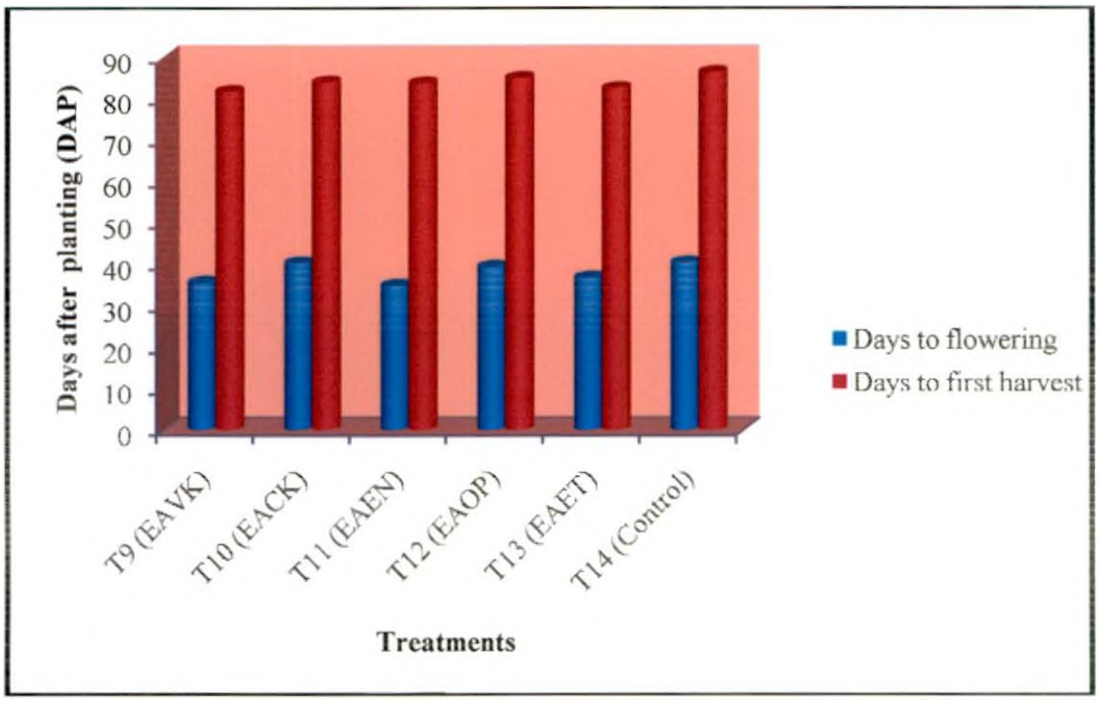


Fig. 4. Effect of endophytic actinomycetes on days to flowering and days to first harvest

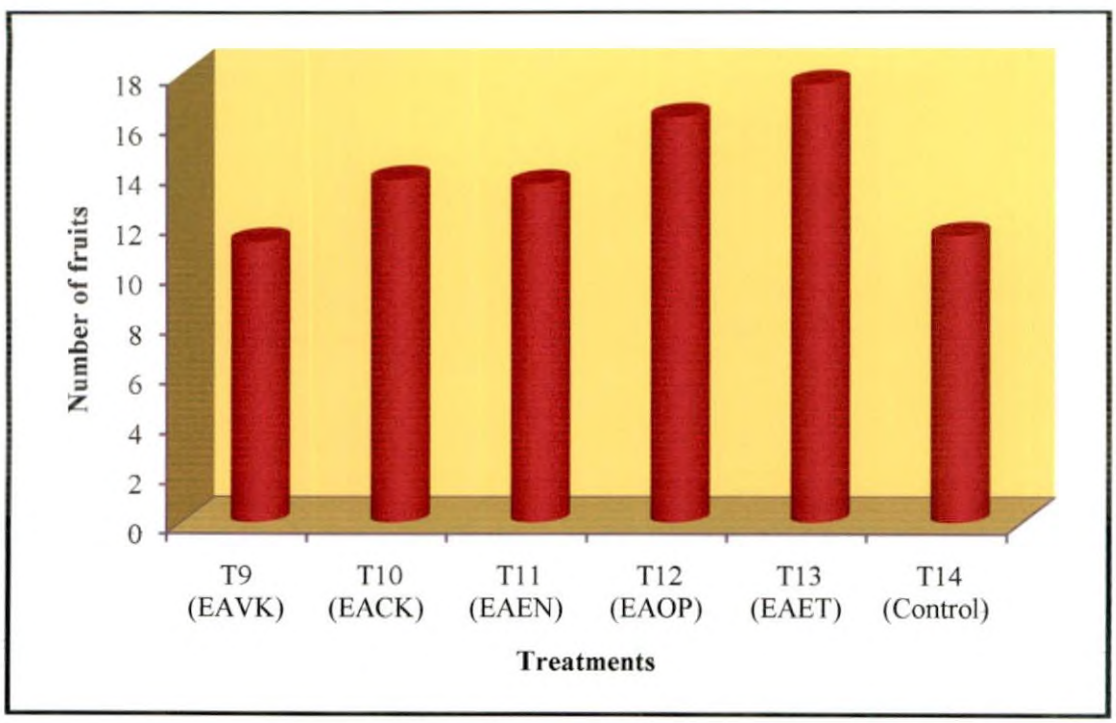
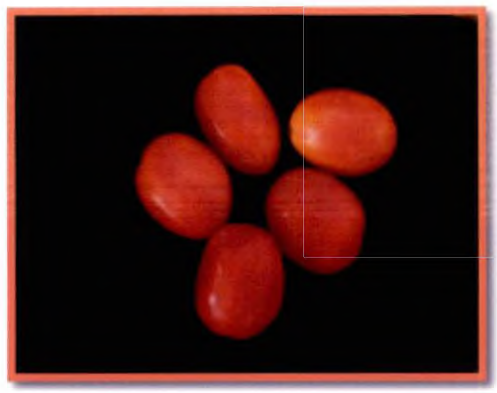


Fig.5. Effect of endophytic actinomycetes on number of fruits

T_{12} (EAOP)



T_{13} (EAET)

Control

Plate 9. Effect of the best two isolates on fruit weight

4.8.8. Yield per plant

The effect of various endophytic actinomycetes on yield per plant showed significant difference among the treatments (Table 12). The maximum yield (332.02 g) was recorded in plants treated with EAET (T_{13}) isolate which was on par with EAOP (T_{12}) isolate (316.81 g). The lowest yield (131.75 g) was recorded in the control plants (Figure 7).

4.9. 16S rRNA SEQUENCE ANALYSIS OF SELECTED ENDOPHYTIC ACTINOMYCETES ISOLATES

Based on the results of pot culture experiment, best three endophytic actinomycetes isolates (EAOP, EAET and EACK) obtained were identified by 16S rRNA sequence analysis using Polymerase Chain Reaction (PCR).

4.9.1. Amplification of 16S rRNA gene

Amplification of 16S rRNA gene was carried out by colony PCR. The PCR product was checked on 0.9% (w/v) agarose gel and documented. Only one amplicon of about 1500bp was obtained (Plate 10).

4.9.2. Direct purification of PCR product

Since only a single band was obtained, PCR product was directly purified.

4.9.3. Sequencing of 16S rRNA gene

Purified product of 1500bp from isolates EAOP, EAET and EACK were sequenced. The nucleotide sequences of the best isolate among the three (EAOP) is given in Appendix- II.

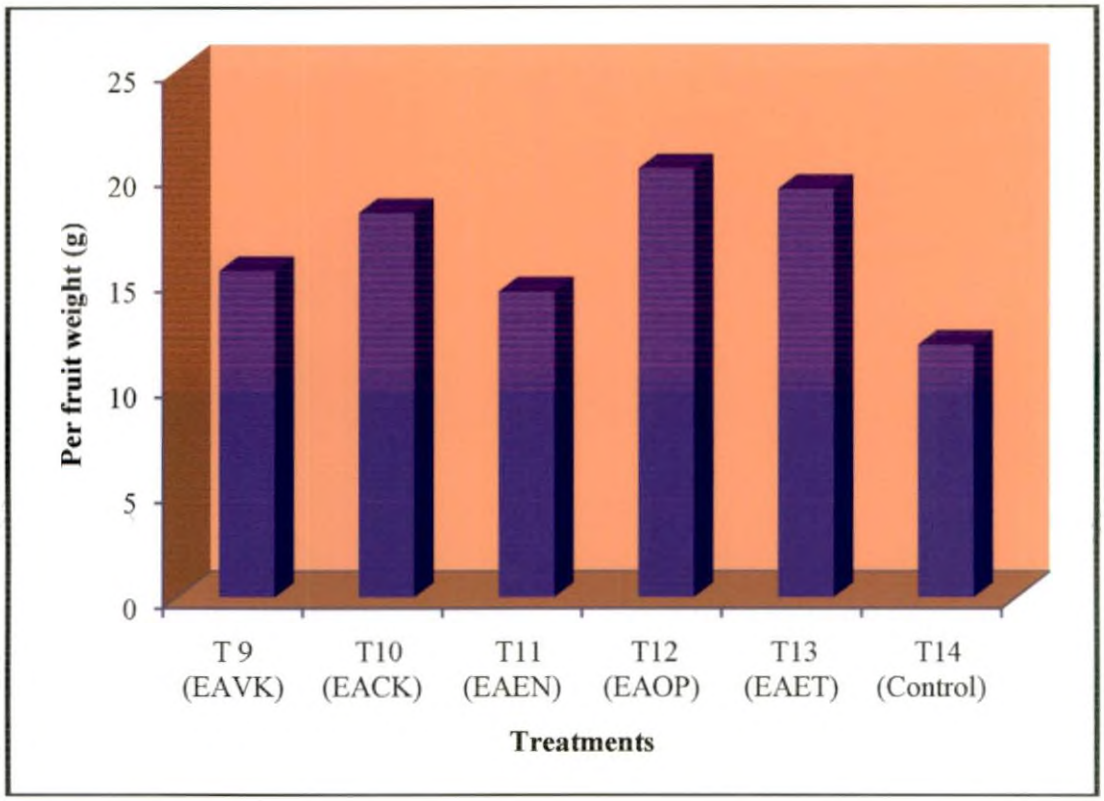


Fig.6. Effect of endophytic actinomycetes on per fruit weight

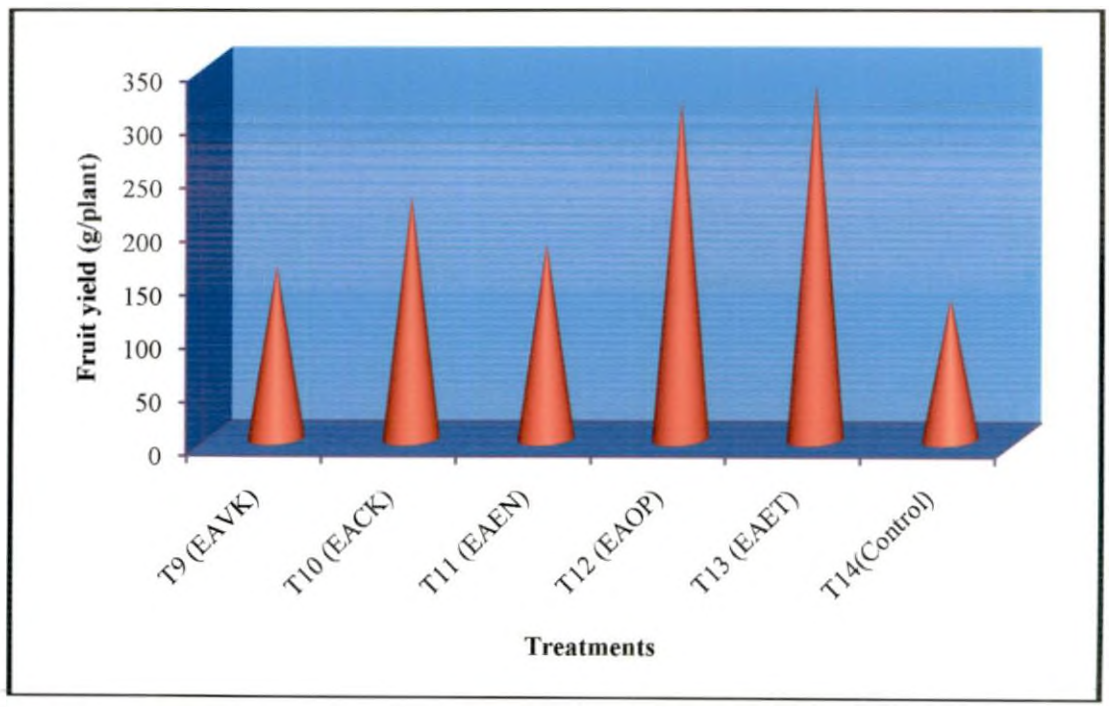


Fig.7. Effect of endophytic actinomycetes on yield

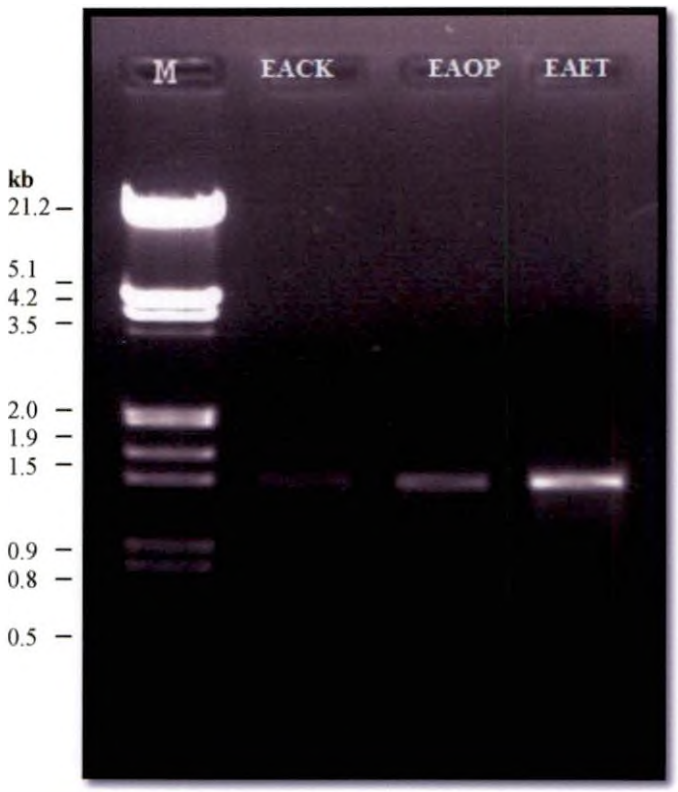


Plate 10. Amplification of 16S rRNA gene of endophytic actinomycetes

EACK -Cherumkuzhy isolate

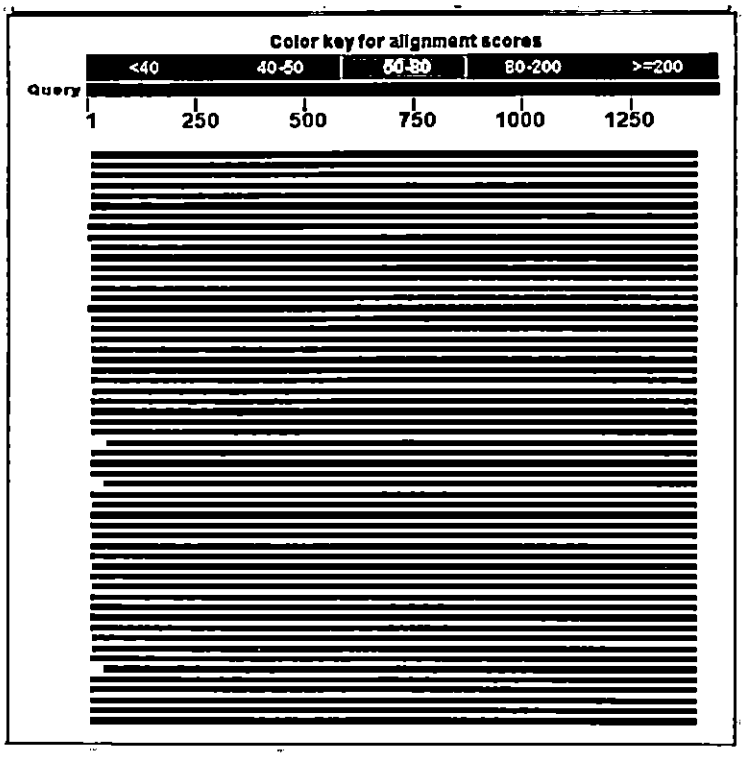
EAOP - Ozhalapathy isolate

EAET - Eruthenpathy isolate

4.9.4. Nucleotide sequence analysis

Homology search of nucleotide sequences obtained from the isolates EAOP, EAET and EACK with other reported 16S rRNA gene sequences was carried out. The best isolate among the three (EAOP), showed homology with *Streptomyces thermodiastaticus* (95% query coverage and 97% identity). The sequence analysis of EAOP isolate is given in Plate 11.

All the isolates were identified by National Center for Fungal Taxonomy (NCFT), New Delhi based on their morphological and cultural characters as *Streptomyces glaucescens* (EAVK) (No.4286.11), *Streptomyces griseoruber* (EACK) (No.4283.11), *Streptomyces griseous* (EAEN) (No.4285.11), *Streptomyces thermodiastaticus* (EAOP) (No.4287.11) and *Streptomyces griseolus* (EAET) (No.4284.11).The identity of three efficient isolates (EAOP, EAET and EACK) obtained from pot culture experiment were further confirmed by the 16S rRNA sequence analysis.



Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AF441160.1	Streptomyces sp. CM-M-1035 16S ribosomal RNA gene, partial se	2420	2420	95%	0.0	98%
GU348992.1	Streptomyces sp. GXT6 16S ribosomal RNA gene, partial sequenc	2414	2414	95%	0.0	98%
AB249966.1	Streptomyces mexicanus gene for 16S rRNA, partial sequence, str	2392	2392	95%	0.0	97%
AF018096.1	Streptomyces thermodiastaticus gene for 16S rRNA, partial seque	2370	2370	95%	0.0	97%
Z68095.1	S.thermoviolaceus 16S rRNA gene	2370	2370	95%	0.0	97%
AB249938.1	Streptomyces thermocoprophilus gene for 16S rRNA, partial seque	2337	2337	95%	0.0	97%
FJ217192.1	Streptomyces sp. BQAB-04d 16S ribosomal RNA gene, partial seq	2335	2335	96%	0.0	97%
EU603350.1	Streptomyces sp. MJM4235 16S ribosomal RNA gene, partial sequ	2335	2335	96%	0.0	97%
FJ217191.1	Streptomyces sp. BQAB-03d 16S ribosomal RNA gene, partial seq	2333	2333	96%	0.0	97%
AB184685.1	Streptomyces thermoviolaceus subsp. spingens gene for 16S rRN	2331	2331	95%	0.0	97%
AB184583.1	Streptomyces thermocyaneomaculatus gene for 16S rRNA, partial	2331	2331	95%	0.0	97%
AB184582.1	Streptomyces thermocyaneoviolaceus gene for 16S rRNA, partial s	2331	2331	95%	0.0	97%
AB184549.1	Streptomyces thermoviolaceus subsp. thermoviolaceus gene for 1	2331	2331	95%	0.0	97%
AB184371.1	Streptomyces thermoviolaceus subsp. thermoviolaceus gene for 1	2331	2331	95%	0.0	97%
AB184194.1	Streptomyces chromofuscus gene for 16S rRNA, partial sequence,	2329	2329	95%	0.0	97%
FJ217193.1	Streptomyces griseoaurantiacus strain BQAB-05d 16S ribosomal R	2326	2326	96%	0.0	96%
NR_027616.1	Streptomyces thermoviolaceus subsp. thermoviolaceus strain DSM	2326	2326	95%	0.0	97%
GU350310.1	Streptomyces ansochromogenes subsp. ansochromogenes strain	2324	2324	95%	0.0	97%
NR_025291.1	Streptomyces thermocoprophilus strain B19 16S ribosomal RNA, p	2324	2324	95%	0.0	96%
EU593556.1	Streptomyces cinereospinus strain 173285 16S ribosomal RNA ge	2322	2322	95%	0.0	97%
FN667129.1	Uncultured compost bacterium partial 16S rRNA gene, clone FS10	2320	2320	95%	0.0	97%
AB184448.2	Streptomyces ansochromogenes subsp. ansochromogenes gene f	2320	2320	95%	0.0	96%
AB249917.1	Streptomyces glomeratus gene for 16S rRNA, partial sequence, st	2320	2320	95%	0.0	96%
DQ462660.1	Streptomyces sp. B198 16S ribosomal RNA gene, partial sequenc	2320	2320	95%	0.0	97%
AB184358.1	Streptomyces thermophilus gene for 16S rRNA, partial sequence,	2320	2320	95%	0.0	96%
AJ781754.1	Streptomyces glomeratus 16S rRNA gene, type strain LMG 19903	2320	2320	95%	0.0	96%
NR_026071.1	Streptomyces thermocarboxydovorans strain AT32 16S ribosomal	2320	2320	95%	0.0	96%
U94487.1	Streptomyces thermocarboxydovorans DSM 44294 16S ribosomal	2320	2320	95%	0.0	96%
EU551708.1	Streptomyces sp. C63 16S ribosomal RNA gene, partial sequence	2318	2318	93%	0.0	97%
EU570681.1	Streptomyces cinereospinus strain 173825 16S ribosomal RNA ge	2318	2318	95%	0.0	97%
DQ442529.1	Streptomyces naganishii strain NRRL B-1816T 16S ribosomal RNA	2318	2318	95%	0.0	96%
AB184224.1	Streptomyces naganishii gene for 16S rRNA, partial sequence, str	2318	2318	95%	0.0	96%

Plate 11. Sequence analysis of EAOP isolate

Discussion

5. DISCUSSION

Tomato is one of the important and remunerative vegetable crops in India. A rich source of minerals, vitamins and organic acids, tomato fruit also provides 3-4% total sugar, 4-7% total solids, 15-30 mg/100g ascorbic acid and 20-50 mg/100g fruit weight of lycopene. Bacterial wilt caused by *Ralstonia solanacearum* is one of the major diseases in tomato and cent per cent yield loss has been reported among the susceptible varieties in our state. *Ralstonia solanacearum* is a genetically diverse soil borne pathogen with a wide host range and is difficult to control the disease once it is established in the field.

Indiscriminate use of chemicals may result in the development of resistant strains of pathogen as well as residual effect on the vegetable. In this context, biological control of the disease is an alternative way. Endophytes are those microorganisms that inhabit for at least one period of their lifecycle inside plant tissues without causing any apparent harm to the hosts. Compared to actinomycetes in the rhizosphere, endophytic actinomycetes are not subjected to competition from soil microbes and so they can be considered as potential biocontrol agents. However its studies on plant growth promotion and management of bacterial wilt in tomato are too little. So the present study was taken up to know about the bioefficacy of endophytic actinomycetes on plant growth promotion and management of bacterial wilt in tomato.

The main aim of the study was to isolate endophytic actinomycetes from healthy tomato plants in Vellanikkara, Cherumkuzhy, Elanad (Thrissur district), Ozhalapathy and Eruthenpathy (Palakkad district). The isolation resulted in maximum number of endophytic actinomycetes from the roots of tomato plants collected from Cherumkuzhy followed by Eruthenpathy. The least count was obtained from the stem of plant sample collected from Vellanikkara and Elanad. This is in accordance with the results obtained by Shimizu *et al.* (2000); Tian *et*

al. (2004) and Verma *et al.* (2009), who reported that more diverse endophytic actinomycetes were isolated from roots than from stem or leaves of host plants. Tan *et al.* (2006) also isolated endophytic actinomycetes from the roots of tomato plants and observed that the proportion of antagonistic strains from healthy plants were higher than from wilted plants. Isolation of endophytic actinomycetes from tomato plants have also been reported by other workers also (Cao *et al.,* 2004b; Teng *et al.,* 2006; Zhiqi *et al.,* 2006; Jiefeng *et al.*, 2009).

The bacterial wilt pathogen isolated from wilted tomato plants on TZC agar medium showed circular, smooth, convex, fluidal, slimy and creamy white colonies with light pink centre after 48 hours of incubation indicating its identity as *Ralstonia solanacearum* (Kelman, 1954).

In vitro evaluation of the antagonistic effect of endophytic actinomycetes against *R. solanacearum* showed maximum inhibition (29.25%) of pathogen with the Vellanikkara isolate (EAVK), followed by the Ozhalapathy isolate (EAOP) (22.59%). Minimum inhibition of the pathogen (8.14%) was observed with the Eruthenpathy isolate (EAET). The antagonistic effect of endophytic actinomycetes against *R.solanacearum* has been reported by other workers as well. Moura *et al.* (1998) observed that endophytic actinomycetes isolated from the root tissues of tomato showed cent percent control of the pathogen. Similarly, Moura and de-Romeiro (1999) noted that endophytic actinomycetes isolated from various hosts showed a high inhibitory activity against *R.solanacearum.*Mathew, (2002) also reported the antagonistic activity of rhizosphere actinomycetes from Ozhalapathy against *R.solanacearum.*

The effect of culture filtrate of endophytic actinomycetes against *R.solanacearum* was also studied. Here, maximum per cent inhibition of the pathogen was recorded by the Cherumkuzhy isolate (EACK) (44.63%) followed by the Ozhalapathy isolate (EAOP) (42.59%). This was in agreement with the

findings of El-Abyad *et al.* (1993) who reported that 80% concentration of culture filtrate of two *Streptomyces* sp. was detrimental to the bacterial population of *R.solanacearum*. Tan *et al.* (2006) reported that many antagonistic *Streptomyces* sp. isolated from tomato roots showed the ability to degrade the cell wall of *R.solanacearum*. Similarly the presence of some lytic enzymes in the culture filtrate may be one of the mechanisms of biocontrol of the isolates in the present study also. Based on the results obtained, EAVK, EACK and EAOP were found to be the efficient isolates with maximum inhibition of pathogen under *in vitro* conditions.

The isolates were identified based on their morphological, cultural and molecular characters. Morphological characters such as presence or absence of substrate and aerial mycelium, fragmentation and sporulation of substrate mycelium, sclerotia formation, and aerial mycelium with chains of arthrospores, spore chain morphology, spore surface ornamentation and motility of spores were studied by light microscopy. Cultural characters such as colony size, color of spore mass, pigmentation of substrate mycelium and diffusible pigment production were studied based on standard keys. Based on the morphological and cultural characters the endophytic actinomycetes were tentatively identified as genus *Streptomyces*. This was in agreement with the results of identification of endophytic actinomycetes from Italian native plants (Sardi *et al.*, 1992), wheat roots from a range of sites across South Australia (Coombs and Franco, 2003) and tomato roots in South China (Cao *et al.*, 2004).

The capacity of isolates in IAA production was assessed qualitatively and quantitatively. All the isolates produced varying levels of IAA ranging from 3.2 to 73.1 µg/ml. The maximum quantity of IAA was produced by the Ozhalapathy (EAOP) isolate and the minimum quantity was produced by the Vellanikkara (EAVK) isolate. Khamna *et al.* (2009) also noticed variation among endophytic actinomycetes isolated from medicinal plants in the production of IAA ranging

from 5.5 to144 µg/ml. IAA production by endophytic actinomycetes has been reported by other workers as well (de-Oliveira *et al.*, 2010; Nimnoi *et al.*, 2010; Suttiviriya *et al.*, 2008).

To understand the various mechanism of action of the endophytic actinomycetes as a biocontrol agent, production of siderophores, hydrogen cyanide, ammonia and non-volatile metabolites were tested based on standard protocols. Iron is a growth limiting factor for majority of organisms and in many cases unavailability of iron results in deleterious effects in their growth. The present study revealed that the isolates produced siderophores and the capacity of endophytic actinomycetes to produce siderophores reduced as the concentration of ferric chloride increased in the broth. Further, there was a variation in the production of siderophores by different isolates. The maximum siderophore was produced by EAOP isolate and the least was produced by EACK isolate, without the addition of ferric chloride. Iron dependent production of siderophores was found to be in agreement with the findings of Nieland (1995), who reported that siderophores are produced under conditions of low iron availability. Tan *et al.* (2006) also reported that the different soil environments of each site probably contained variant concentrations of available iron and also the ability of endophytic *Streptomyces* isolated from different sites to absorb available iron varied.

Production of volatile and non volatile metabolites plays an important role in the antagonistic activity of bioagents against pathogens. With regard to the production of hydrogen cyanide, it was noticed that all the isolates tested were non cyanogenic in nature. Such inability endophytic actinomycetes to produce HCN had been noticed by Malviya *et al.* (2009). The ability of isolates for the production of ammonia, a volatile compound having a direct bearing on the biocontrol activity was tested. It was found that the isolates EAVK, EACK and EAET produced the maximum amount of ammonia and it might be the one of the

mechanism of antagonism of theses isolates under *in vitro*. The isolate EAOP produced the least amount. Nimnoi *et al.* (2010) also reported the production of ammonia by endophytic actinomycetes isolated from healthy shoots and roots of *Aquilaria crassna* Pierre ex Lec (eagle wood). Non- volatile metabolites were produced by all the isolates tested which was confirmed by the complete inhibition in the pathogen's growth. Evidences for the production of non volatile metabolites by endophytic actinomycetes had been established by El- Tarabily *et al.* (1997) who reported that streptomycetes and non-streptomycetes actinomycetes isolated from carrot rhizosphere produced non- volatile antifungal metabolites.

Finally the effect of endophytic actinomycetes on plant growth promotion and management of bacterial wilt in tomato was assessed under pot culture conditions. Significant difference existed among various treatments studied with respect to per cent bacterial wilt incidence. The minimum per cent wilt incidence was shown by plants in pots treated with a mixture of urea (44 g m^{-2}) and lime (500 g m^{-2}) (29.63%). The results were in accordance with the findings of Mathew (2004), who reported the effectiveness of urea (44 g m^{-2}) and lime (500 g m^{-2}) mixture for the management of *R. solanacearum* infecting solanaceous vegetables.

Kanjilal *et al.* (2000) also reported that significant decrease in wilt occurrence was observed following the treatment of soil with urea, fly ash and bleaching powder singly or in combination. Among the endophytic actinomycetes tested, the plants treated with EAOP isolate showed the minimum per cent wilt incidence followed by plants in pots drenched with 0.2% copper hydroxide (40.74%). The effect of copper hydroxide in reducing the wilt incidence was in conformity with the findings of Akbar (2002) and Mathew (2002), who reported that soil drenching with 0.15% copper hydroxide at the time of planting and 30 days after planting is effective in reducing the wilt incidence of tomato.

Minimum per cent wilt index was also shown by plants in pots treated with urea (44 g m^{-2}) and lime (500 g m^{-2}) (T_6) (25.92 %) and it differed significantly from the other treatments. Among the endophytes, plants treated with EAOP (T_4) isolate (36.30 %) recorded the minimum per cent wilt index. It was observed from the present study that application of either a mixture of urea (44 g m^{-2}) and lime (500 g m^{-2}) or EAOP isolate were the equally best treatments in the management of bacterial wilt in tomato.

To evaluate the performance of endophytic actinomycetes in plant growth promotion, observations on biometric characters such as plant height, days to flowering, days to first harvest, number of fruits per plant, per fruit weight and yield per plant were taken. There was significant difference among the treatments with respect to plant height from 60 DAP. The height was found maximum in plants treated with EAOP isolate followed by EAET isolate and the minimum plant height was observed in plants treated with EACK isolate. The reason behind the improved performance of EAOP and EAET might be the production of higher levels of IAA, which is a growth hormone. This was in concordance with the findings of Moura and Romeiro (2000). Igarashi *et al.* (2005) also reported that a strain of *Streptomyces hygroscopicus* S-17 induced significant increase in plant height of tomato compared to control.

With respect to yield parameters such as days to flowering, days to first harvest, per fruit weight and yield per plant, plants treated with the endophytic actinomycetes were superior in performance when compared to the control. The number of days taken for the emergence of first flower and also the number of days taken from fruit set to harvest should be less so that the total crop duration is not extended unduly. In the present study the plants treated with EAEN isolate recorded the minimum number of days (34.83) for flowering and the plants treated with EAVK took the minimum number of days (81.66) to first harvest.

Number of fruits per plant and per fruit weight are the two parameters having a direct effect on crop yield. In the present study, the plants treated with EAET isolate produced the maximum number of fruits per plant and the plants treated with EAVK isolate produced the least amount. Regarding per fruit weight, the plants treated with EAOP isolate produced fruits with maximum weight followed by EAET isolate. The effect of endophytic actinomycetes on yield per plant also showed significant differences among the treatments. Highest yield was recorded for the plants treated with EAET isolate followed by EAOP isolate. Evidences of plant growth promoting activities of endophytic actinomycetes had been well documented by several workers (Moura and Romeiro, 2000; Igarashi *et al.;* 2005; Meugro *et al.,* 2006; Franco *et al.,* 2006; El-Tarabily 2008; Hasegawa *et al.,* 2008). It was concluded from the study that both EAOP and EAET were the efficient isolates in plant growth promotion.

After the evaluation of endophytic actinomycetes against *R.solanacearum* under pot culture experiment, three efficient isolates that showed minimum per cent wilt incidence and index were selected and 16S rRNA sequence analysis of those three isolates were carried out. The amplification of 16S rRNA gene using colony PCR yielded only one amplicon of about 1500 bp. The purified PCR product from the three isolates was sequenced using the primers 27f and 1492r. Homology search of nucleotide sequences obtained from the isolates with other reported 16S rRNA gene sequences was carried out and they were identified up to the species level. This was in conformity with the findings of Cook and Meyers (2003). They amplified nearly full length of 16S rDNA sequences of filamentous actinomycetes using primers F1 and R5. Primer F1 bound to the base positions 7-26 and primer R5 to the base positions the 1496-1476 of the 16S rRNA gene of *Streptomyces ambofaciens.*

All the isolates were identified up to the species level by National Center for Fungal Taxonomy (NCFT), New Delhi based on their morphological and cultural characters as *Streptomyces glaucescens* (EAVK), *Streptomyces griseoruber* (EACK), *Streptomyces griseous* (EAEN), *Streptomyces thermodiastaticus* (EAOP) and *Streptomyces griseolus* (EAET) The identity of three efficient isolates (EAOP, EAET and EACK) obtained under pot culture experiment were further confirmed by the 16S rRNA sequence analysis.

It was observed that maximum inhibition of *R. solanacearum* was recorded with EAVK and EAOP isolate under *in vitro* conditions. All the isolates were able to produce IAA, siderophores, ammonia and non- volatile metabolites. None of the isolates were found to be cyanogenic in nature. The isolates EAOP and EAET were the best plant growth promoters. Applications of either a mixture of urea (44 gm^{-2}) and lime (500 gm^{-2}) or EAOP 4 isolate showed the best suppressive effect of the bacterial wilt pathogen. So it may be concluded from the present study that EAOP (*Streptomyces thermodiastaticus*) was the highly promising endophytic actinomycete isolate in plant growth promotion as well as in the management of bacterial wilt in tomato.

In future, the efficiency of Ozhalapathy isolate (EAOP) can be tested by conducting field trials and also multi- locational trials. The possibility of commercialization of this isolate also may be explored.

Summary

6. SUMMARY

The present investigation on "Bioefficacy of endophytic actinomycetes on plant growth promotion and management of bacterial wilt in tomato" were undertaken in the Department of Plant Pathology, College of Horticulture, Vellanikkara during 2009-11 with an aim to isolate endophytic actinomycetes from healthy tomato plants, to study the antagonistic effect of these endophytic actinomycetes against bacterial wilt pathogen under *in vitro* conditions and their evaluation against bacterial wilt pathogen under pot culture experiment. The salient findings of the study are summarized below:

- The maximum population of endophytic actinomycetes was obtained from the roots of tomato plant collected from Cherumkuzhy (EACK) ($8x10^1$ cfu/g), followed by Eruthenpathy (EAET) ($3x10^1$ cfu/g). The least count was obtained from Vellanikkara (EAVK), Elanad (EAEN) and Ozhalapathy (EAOP) ($1x10^1$cfu/g). More number of endophytic actinomycetes was obtained from roots than from stem of tomato plants.

- The pathogen causing bacterial wilt in tomato was circular, smooth, convex, slimy, fluidal, creamy white colonies with light pink centre indicating its identity as *Ralstonia solanacearum.*

- The antagonistic effect of endophytic actinomycetes against *R. solanacearum* was evaluated under *in vitro*. The maximum inhibition (29.25%) of pathogen was recorded by the Vellanikkara isolate (EAVK), followed by the Ozhalapathy isolate (EACK) (22.59%). The least inhibition (8.14%) of the pathogen was recorded by the Eruthenpathy isolate (EAET).

- The effect of culture filtrate of endophytic actinomycetes against *R.solanacearum* was also noted. Here, maximum inhibition (44.63%) of

the pathogen was recorded by the Cherumkuzhy isolate (EACK), followed by the Ozhalapathy isolate (EAOP) (42.59%). The least inhibition (26.11%) was shown by the Elanad isolate (EAEN). Based on the dual culture method and the effect of culture filtrate of the isolates, EAOP was found to be the best isolate under *in vitro* conditions.

- The endophytic actinomycetes were identified based on their morphological and cultural characters, indicating that the isolates belonged to genus *Streptomyces.*

- All the isolates produced varying levels of IAA ranging from 3.2 to 73.1 µg/ml. The maximum quantity of IAA was produced by the Ozhalapathy (EAOP) isolate and the minimum quantity was produced by the Vellanikkara (EAVK) isolate.

- All isolates tested produced siderophores and the capacity of isolates to produce the same, reduced as the concentration of ferric chloride increased in the broth. The maximum siderophore was produced by EAOP isolate and the least was produced by EACK isolate, without the addition of $FeCl_3$. $6H_2O$. With regard to the production of hydrogen cyanide, it was noticed that all the isolates tested were non cyanogenic in nature.

- It was found that the isolates EAVK, EACK and EAET produced the maximum amount of ammonia, a volatile compound having a direct bearing on the biocontrol activity. The isolate EAOP produced the least amount. Non- volatile metabolites were produced by all the isolates tested which was confirmed by the complete inhibition in the pathogen's growth.

- The effect of endophytic actinomycetes on plant growth promotion and management of bacterial wilt in tomato was assessed under pot culture conditions. The minimum per cent wilt incidence was shown by plants in pots treated with a mixture of urea (44 g m^{-2}) and lime (500 g m^{-2})

(29.63%). Among the endophytes, the plants treated with EAOP (T_4) isolate showed minimum per cent wilt incidence (37.03%). The minimum per cent wilt index was also recorded with urea (44 g m^{-2}) and lime (500 g m^{-2}) (25.9%), followed by EAOP isolate (36.3%). It was observed from the present study that application of a mixture of urea + lime or EAOP isolate were the best treatments in the management of bacterial wilt in tomato.

- The plant height was found maximum in plants treated with EAOP isolate followed by EAET isolate. The plants treated with EAEN isolate took the minimum number of days for flowering where as the plants treated with EAVK isolate took the minimum number of days to first harvest.The plants treated with EAET isolate produced the maximum number of fruits per plant and the plants treated with EAOP isolate produced fruits with maximum weight. Highest yield was recorded for the plants treated with EAET isolate and the lowest yield was for the control plants. It was observed from the study that EAOP and EAET were the efficient isolates in plant growth promotion.

- 16S rRNA sequence analysis was carried out for three efficient isolates (EAOP, EAET and EACK) obtained from pot culture experiment that showed minimum per cent wilt incidence and index. The amplification of 16S rRNA gene using colony PCR yielded only one amplicon of about 1500 bp.

- The isolates were identified up to the species level by National Center for Fungal Taxonomy (NCFT), New Delhi based on their morphological and cultural characters as *Streptomyces glaucescens* (EAVK), *Streptomyces griseoruber* (EACK), *Streptomyces griseous* (EAEN), *Streptomyces thermodiastaticus* (EAOP) and *Streptomyces griseolus* (EAET). The identity of three efficient isolates (EAOP, EAET and EACK) obtained

under pot culture experiment were further confirmed by the 16S rRNA sequence analysis and were found to be the same as *Streptomyces thermodiastaticus, Streptomyces griseolus* and *Streptomyces griseoruber.*

- The present study clearly indicated that the Ozhalapathy isolate (EAOP) (*Streptomyces thermodiastaticus*) was the most efficient among the five isolates of endophytic actinomycetes in plant growth promotion as well as in the management of bacterial wilt in tomato.

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Appendices

APPENDIX – I

1. Triphenyl tetrazolium chloride media (TZC) (pH 6.8)

Peptone	- 10.0 g
Casein hydrolysate	- 1.0 g
Glucose	- 5.0 g
Agar	- 20.0 g
Distilled water	- 1000 ml

Autoclave 5 ml of 1% triphenyl tetrazolium chloride

2. Nutrient agar media (NA) (pH 7.2)

Peptone	- 10.0 g
Beef extract	- 5.0 g
NaCl	- 3.0 g
Agar	- 20.0 g
Distilled water	- 1000 ml

3. Kenknights agar media (KAM) (pH 7.0)

Dextrose	- 20.0 g
KH_2PO_4	- 0.1 g
$NaNO_3$	- 0.1 g
KCl	- 0.1 g
$MgSO_4.7H_2O$	- 0.1 g
Agar	- 20.0 g
Distilled water	- 1000 ml

4. Potato dextrose agar media (PDA) (pH 7.0)

Potato	- 200 g
Dextrose	- 20 g
Agar	- 20 g
Distilled water	- 1000 ml

5. Peptone water (pH 7.0)

Peptone	- 10.0 g
NaCl	- 15.0 g
Distilled water	- 1000 ml

6. Yeast extract – Malt extract agar media (YM) (pH 7.0)

Yeast extract	- 4.0 g
Malt extract	- 10.0 g
Glucose	- 4.0 g
Agar	- 20.0 g
Distilled water	- 1000 ml

APPENDIX – II

Nucleotide sequences of EAOP isolate

TATTGCAAGCGCAGCTTACAATGCAGTCGTACCATGGATACCCGTTCGGGAG
GGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTG
GGACAAGCCCTGGAAACGGGGTCTAATACCGGATACGACTCAGGACCGCAT
GGTCTCTGGGTGGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAG
CTTGTTGGTGAGGTAACGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAG
AGGGCGACCGGCCACACTGGGACTAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCG
CGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGC
GAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAG
CCGTAGGCGGCTTGTCGCGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGT
CGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGG
TGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGG
ATCTCTGGGCCGATACTGACGCTGAGGAGCGAAGCGTGGGGAGCGAACAGG
ATTAGATACCCTGGTAGTTCCACGCCGTAACCGGTGGGCCATTAGTTGTGGGC
AACATTCCACCGTTGTCCGTGCCGCAGCTAACGCCATTAAGTGCCCTGCTTGG
GGAGTACGGGCCGCAAGGCTAAATCTCAAGGAATTTGACGGGGGCCCGCAC
AAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAG
GCTTGACATACACGGAAACATCCAGAGATGGGTGCCCCCTTGTGGTCGGTGT
ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC
CCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAGGCCCTTGTGGGCTG
GGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACG
TCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGG
TACAATGAGCTGCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTC
AGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTATAAT
CGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACGCC
CGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCTGTAGTTGATCC
TGGCTCAGGACGAATAGCGACGACGCAAGGCTA

BIOEFFICACY OF ENDOPHYTIC ACTINOMYCETES ON PLANT GROWTH PROMOTION AND MANAGEMENT OF BACTERIAL WILT IN TOMATO

By

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ABSTRACT OF THE THESIS

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ABSTRACT

The present study on "Bioefficacy of endophytic actinomycetes on plant growth promotion and management of bacterial wilt in tomato" was undertaken in the Department of Plant Pathology, College of Horticulture, Vellanikkara during 2009-11.The main objectives were to isolate endophytic actinomycetes from healthy tomato plants collected from five different locations, to study the antagonistic effect of those endophytic actinomycetes against bacterial wilt pathogen under *in vitro* conditions and their evaluation against bacterial wilt pathogen under pot culture experiment.

Endophytic actinomycetes were isolated from tomato plants in Vellanikkara, Cherumkuzhy, Elanad (Thrissur district), Ozhalapathy and Eruthenpathy (Palakkad district). Only a single type of isolate was obtained from the different locations. The population was maximum in the roots of sample collected from Cherumkuzhy (EACK) ($8x10^1$ cfu/g) followed by Eruthenpathy (EAET) ($3x10^1$ cfu/g). The least count of endophytic actinomycetes was obtained from Vellanikkara (EAVK), Elanad (EAEN) and Ozhalapathy (EAOP) ($1 x10^1$ cfu/g). The pathogen causing bacterial wilt in tomato was circular, smooth, convex, slimy, fluidal, creamy white colonies with light pink centre indicating its identity as *Ralstonia solanacearum.*

The efficacy of endophytic actinomycetes against *R.solanacearum* was evaluated under *in vitro* conditions. Out of the five isolates, maximum inhibition of the pathogen was observed with the Vellanikkara isolate (EAVK) (29.25%) which was on par with Ozhalapathy isolate (EAOP) (22.59%). The efficacy of culture filtrate of endophytic actinomycetes against *R.solanacearum* was also evaluated under *in vitro* conditions. Among the isolates, maximum inhibition of the pathogen was recorded with the Cherumkuzhy isolate (EACK) (44.63%) which was on par with Ozhalapathy isolate (EAOP) (42.59%). Based on the dual culture method and the effect of culture filtrate of antagonists, EAOP was found

to be the best isolate under *in vitro* conditions. The morphological and cultural characters of endophytic actinomycetes were studied based on standard keys and the isolates were tentatively identified as *Streptomyces*.

Among the isolates, EAOP produced the maximum concentration of IAA (73.1 µg/ml) followed by EACK (41.8 µg/ml) and the least was produced by EAVK (3.2 µg/ml). The mechanism of action of endophytic actinomycetes was studied based on the production of siderophores, HCN, ammonia and non-volatile metabolites by the isolates. The siderophore production was maximum in EAOP isolate followed by EAVK isolate and the least was produced by EACK isolate. None of the isolates were able to produce hydrogen cyanide. Maximum ammonia production was shown by EAVK, EACK and EAET isolates. All the isolates were able to produce non- volatile metabolites.

Evaluation of endophytic actinomycetes against bacterial wilt pathogen was carried out under pot culture conditions. Minimum per cent wilt incidence was shown by plants in pots treated with urea (44 g m^{-2}) and lime (500 g m^{-2}) (T_6) (29.63%) and it differed significantly from the other treatments. Among the endophytes, the plants treated with EAOP (T_4) isolate showed minimum per cent wilt incidence (37.03%). The minimum per cent wilt index was also recorded with urea and lime (25.9%), followed by EAOP isolate (36.3%). Among the endophytic actinomycetes, EAOP was the most promising isolate for the management of bacterial wilt in disease.

The efficiency of endophytic actinomycetes in plant growth promotion was assessed under pot culture experiment. The plant height was maximum in plants treated with EA OP isolate (77.31 cm). The plants treated with EAEN isolate took the minimum number of days for flowering (34.83 DAP) where as the plants treated with EAVK isolate took the minimum number of days to first harvest (81.66 DAP). Plants treated with EAET isolate produced the maximum number of fruits per plant (17.57) and the plants treated with EAOP isolate produced fruits

with maximum weight (20.30 g). Highest yield was recorded for the plants treated with EAET isolate (332.02 g/plant) and the lowest yield was for the control plants. It was observed from the study that EAOP and EAET were the promising isolates in plant growth promotion.

The five isolates were identified by National Center for Fungal Taxonomy (NCFT), New Delhi, based on their morphological and cultural characters as *Streptomyces glaucescens* (EAVK), *Streptomyces griseoruber* (EACK), *Streptomyces griseous* (EAEN), *Streptomyces thermodiastaticus* (EAOP) and *Streptomyces griseolus* (EAET). The identity of three efficient isolates (EAOP, EAET and EACK) obtained under pot culture experiment were further confirmed by 16S rRNA sequence analysis using PCR.

The present studies indicated that the Ozhalapathy isolate (EAOP) (*Streptomyces thermodiastaticus*) was the most efficient among the five isolates of endophytic actinomycetes in plant growth promotion as well as in the management of bacterial wilt in tomato.