MICROBIAL ANTAGONISTS AND RESISTANCE INDUCERS FOR THE MANAGEMENT OF BACTERIAL BLIGHT OF ANTHURIUM

(*Anthurium andreanum* Linden)

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DECLARATION

I hereby declare that this thesis entitled "**Microbial antagonists and resistance inducers for the management of bacterial blight of anthurium (*Anthurium andreanum* Linden)**" is a bonafide record of research work 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, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that this thesis entitled "**Microbial antagonists and resistance inducers for the management of bacterial blight of anthurium (*Anthurium andreanum* Linden)**" is a record of research work done independently by Ms. Anjana R. Nair (2003-11-19) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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Dedicated to
My Family

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Anjana R. Nair

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

µg	Micro gram
µl	Micro liter
^{0}C	Degree Celsius
CD	Critical difference
cfu	Colony forming units
cm	Centimeter
et al.	And others
f.sp.	Forme species
Fig.	Figure
g	Gram
h	Hour
i.e.	That is
lbs	Pounds
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
N	Normal
nm	Nanometer
ppm	Parts per million
pv.	Pathovar
rpm	Rotations per minute
S	Seconds
spp.	Species
Var.	Variety
viz.	Namely
Vol	Volume

LIST OF APPENDIX

Sl. No.	Title	Appendix No.
1	Buffers for enzyme analysis	I

Introduction

1. INTRODUCTION

Anthurium is an important cut-flower crop which commands a significant economic share in the cut flower industry of Kerala. *Anthurium andreanum* Linden is the most widely cultivated species. It is a native of South West Colombia and is a common inhabitant of regions extending from Central South America to Central Mexico and Caribbean islands. In India also they come up well. Kerala enjoys an agro climatic condition which is ambient for the cultivation of anthurium. Moreover the cut-flowers have a good clientele within the state and abroad.

The growth of anthurium is compromised by a number of diseases. the serious one being the bacterial blight incited by *Xanthomonas axonopodis* pv. *dieffenbachiae*. The disease was first reported in Brazil in 1960. This disease is not restricted to leaves or spathes. It can advance systemically and kill the entire plant. This disease was first reported in Kerala in 2000 and it was observed that the disease caused considerable economic damage to the farmers (Dhanya, 2000).

The current management practices in Kerala involve the use of antibiotics. But the disease is still looming large over the cut flower industry. This is attributed to the emergence of resistant strains of the pathogen to the chemicals. As an alternate strategy to manage the disease, microbial antagonists and chemical activators can be exploited. Now-a-days management using antagonistic microbes is gaining momentum as they are eco-friendly, cost effective and best suited for greenhouse crops like anthurium. Chemical activators on the other hand act on the host plants. They elicit defense mechanisms in the host plants and there by equip the plants to defend themselves from the pathogen. Unlike the conventional chemicals, the activators do not act directly on the pathogen. thus eliminating any chances of development of resistance by the

pathogen. It would be interesting to study the efficacy of these two strategies in comparison with the existing management practices. With this in perspective, an investigation was undertaken with the following objectives.

1. Isolation of the causal organism of bacterial blight from infected anthurium plants and selecting the most virulent isolate.
2. Isolation of microbial antagonists from rhizosphere and phyllosphere of healthy anthurium plants
3. Testing the antagonistic activity of the isolates against *X. axonopodis* pv. *dieffenbachiae in vitro.*
4. Characterization and identification of the isolates showing maximum antagonism against the pathogen.
5. Testing the chemical activators against *X. axonopodis* pv. *dieffenbachiae in vitro.*
6. Testing the effect of antagonists, chemical activators, botanicals and an antibiotic under green house conditions.

Review of Literature

2. REVIEW OF LITERATURE

2.1 BACTERIAL BLIGHT AND ITS DISTRIBUTION

Bacterial blight incited by *Xanthomonas axonopodis* pv. *dieffenbachiae* (Mc Culloch and Pirone) Vauterin *et al.* is a major concern in the production of anthurium. Mc Culloch and Pirone (1939) first described the disease on Dieffenbachia and named the pathogen as *Bacterium diffenbachiae*. Hayward (1972) first reported bacterial blight of anthurium as a foliar disease from the islands of Kauai, Hawaii. Since then this disease has been reported worldwide from Venezuela (Guevara and Debrot, 1984), California (Cooksey, 1985), Florida (Chase and Poole, 1986), French West Indies (Rott and Prior, 1987), Tahiti (Mu, 1990), Philippines (Natural, 1990), Jamaica (Young, 1990), Reunion Island (Soustrade *et al.*, 2000) and China (Ji *et al.*, 2004).

In India, bacterial blight symptoms were noticed on anthurium plants imported from Netherlands (Satyanarayana *et al.*, 1998). Anthurium blight was also reported from Kerala on varieties imported from abroad. The pathogen isolated was identified as *Xanthomonas* spp. by Das *et al.* (1996) and as *X. axonopodis* pv. *dieffenbachiae* by Dhanya (2000).

2.2 BIOLOGICAL CONTROL

Biological control as a concept and approach to the control of plant pathogens has been defined as the use of natural or modified organisms, genes or gene products to reduce the effect of diseases (Cook, 1989).

2.2.1 Resident Microflora as Biocontrol Agents

In wheat variety Apex, resistant to root-rot caused by *Cochliobolus sativus*, the number of fungi in the rhizosphere was 3,43,000 g^{-1} as against 1,24,000 g^{-1} in the susceptible variety S165. Further 20 per cent of bacterial populations in rhizosphere of Apex were antagonistic to the

pathogen but none in S165 (Neal *et al.*, 1970). A population level of more than 10^6 cfu g^{-1} of rhizosphere soil is necessary for getting consistent control of *Rhizoctonia* in radish (Baker and Chet, 1982). Beagle-Ristaino and Papavizas (1985) observed that the propagules of *Trichoderma harzianum* and *Gliocladium virens* were able to survive and proliferate both in soil and bean rhizosphere.

Bacterization of cotton seeds with antagonistic *Bacillus* spp., *B. subtilis* and *Pseudomonas fluorescens* isolated from cotton leaves were found to be inhibitory towards *Xanthomonas axonopodis* pv. *malvacearum in vitro* (Arya and Parashar, 1999). Borah *et al.* (1999) established as many as 110 bacterial cultures from the phylloplane of mung bean and out of this five isolates were antagonistic to *X. campestris* pv. *vigna-radiata* giving inhibition zones ranging from 6 mm – 22 mm. Jagadeesh and Kulkarni (2003) reported that all the 38 rhizobacteiral strains isolated from tomato were antagonistic to *Ralstonia solanacearum*. A total of 11 out of 538 rhizobacteiral strain isolated from roots of radish seedlings grown in compost amended mixes suppressed bacterial leaf spot of radish (Krause *et al.*, 2003). According to Meenakumari *et al.* (2003) native isolates of fluorescent pseuodmonads are highly potential biocontrol agents capable of suppressing bacterial wilt in chilli and tomato. Out of 600 rice associated bacteria, five strains of *P. fluorescens*, 10 strains of *Bacillus* and an *Enterobacter*, afforded 50-64 per cent suppression of bacterial blight (Velusamy *et al.*, 2003).

2.2.2 Bacterial Antagonists

Bacteria having potential for biocontrol occur in many genera including *Pseudomonas* (Burr *et al.*, 1978), *Agrobacterium* (Kerr, 1980), *Serratia* (Sneh, 1981), *Bacillus* (Capper and Campbell, 1986) etc. A few bacteria, *Agrobacterium radiobacter* strain 84, *Bacillus subtilis* A 13 and *Pseuodmonas fluorescens* 2-79 were proved to be commercially viable

biocontrol agents against certain soil borne plant pathogenic microorganisms under field condition (Weller, 1988).

Agrobacterium radiobacter 84 was the first bacterium which was used commercially as biocontrol agent and it was successful worldwide. This organism has been found to control effectively the crown gall of stone fruit caused by *Agrobacterium tumefaciens* (Moore and Warren, 1979).

Bacillus subtilis A 13 was originally isolated from lysed mycelium of *Sclerotium rolfsii* of peanut by Broadbent *et al.* (1971) which suppressed several major and minor soil borne pathogens. It has been sold under the name Quantum-4000 for seed treatment of peanut in USA (Weller, 1988). Arya and Parashar (1998) reported that *Bacillus subtilis* gave largest inhibition zone around *Xanthomonas axonopodis* pv. *malvacearum in vitro*. Arya and Parashar (1999) proved that bacterization of cotton seeds with antagonistic *Bacillus* sp. and *B. subtilis* resulted in reduced disease incidence. Hong *et al.* (1999) observed that *B. subtilis* B56 showed antagonistic activity against *X. oryzae* pv. *oryzae*. Dipping of potato tubers in bacterial suspensions of *Bacillus* sp., *B. cereus* and *B. subtilis* significantly increased the yield and inhibited wilt incidence and development of brown rot caused by *Ralstonia solanacearum* during harvest and fifty days after storage (Singh and Rana, 2000). Pradeepkumar and Sood (2001) attributed the significant reduction in bacterial wilt incidence in tomato to the incorporation of antagonistic rhizobacteria (*Pseudomonas fluorescens* and *B. cereus*) in soil prior to solarization as the population of the antagonists was considerably enhanced after solarization whereas that of *R. solanacearum* was significantly reduced. Seedling of pepper (Bian Jiao No. 5) were sprayed with bacterial suspension of J_3 (*Pseudomonas* spp.), BB11 and FH17 (*Bacillus* spp.) and admixture of these strains (1 : 1 : 1) of final concentration of approximately 10^{10} cfu ml^{-1} and all these treatments significantly reduced

the bacterial wilt disease, the disease incidence and increased pepper yield (Hua *et al.*, 2002). The biocontrol efficiency of an antagonistic endophytic strain of *B. subtilis* (Strain BB) was evaluated by Wuff, *et al.* (2002) against three strains of *X. campestris* pv. *campestris* causing black rot of brassicas and strain BB controlled the disease. The bacterial strain X2-23 identified as *B. globisporus* showed significant antagonistic effect on *X. oryzae* pv *oryzae* (Hong *et al.*, 2003). Laux *et al.* (2003) tested three bacterial antagonists; strain Pa 21889 of *Pantoea agglomerans*, strain BS BD 170 of *B. subtilis* and strain Ra 39 of *Rahnella aquatilis* against the blossom blight pathogen of apple, *Erwinia amylovora* and the result indicated that all the antagonistic strains significantly reduced the disease (43-81%). Park *et al.* (2003) observed that treatment of *B. amyloliqueifaciens* strain EXTN-1 gave a broad controlling spectrum to major plant diseases caused by viral, bacterial and fungal pathogens as well as plant growth promotion. Isolate 210 identified as *B. subtilis* showed the highest degree of antibiosis against *X. campestris* pv. *glycines* (Salerno and Sagardoy, 2003). The biocontrol efficacy of strains of *B.cereus, B. lentimorbus* and *B. pumilis* was tested against black rot pathogen of cabbage, *X. campestris* pv. *campestris* and it was observed that both incidence and severity were greatly reduced especially when applied through roots (Massomo *et al.*, 2004).

Fluorescent *Pseudomonas* spp. have emerged as the largest and potentially the most promising group of plant growth promoting rhizobacteria (PGPR) which can effectively control many soil borne plant pathogens. Strains of fluorescent *Pseudomonas* have long reigned to be the foremost among biological control agents as they improve plant growth by suppressing either major or minor pathogens of plants (Cook and Rovira, 1976; Weller, 1988; Defago *et al.*, 1990). *P.fluorescens* biotype C and G isolated from the rhizosphere were inhibitory to *Xanthomonas oryzae* pv. *oryzae* (Sakthivel *et al.*, 1986). Anuratha and Gnanamanickam (1987) showed that bacterized plants recorded a substantial reduction (40–60 %) in bacterial blight severity.

Sivamani *et al.* (1987) obtained strains of *P. fluorescens* antagonistic to the bacterial blight pathogen, *X. oryzae* pv. *oryzae*. The antagonistic effect of fluorescent pseudomonads on related species of the pathogen *Xanthomonas* infecting other graminaceous fodder crops, citrus, cotton and cassava have been reported (Unnamalai and Gnanamanickam, 1984; Verma *et al.*, 1986: Schmidt, 1988). A fluorescent pseudomonad, *P. acidovorus* was reported to have the ability to control bacterial blight disease in rice (Sindhan *et al.*, 1997). Magabala (1999) isolated strains of *Pseudomonas* antagonistic to *X. axonopodis* pv. *phaseoli*. *P. fluorescens* when seed bacterized reduced the disease intensity of bacterial blight in cotton (Mondal *et al.*, 2000). Vidhyasekharan *et al.* (2001) observed that seed treatment along with foliar spray of talc based formulation of Pf-1 reduced the disease intensity of bacterial blight (*X. oryzae* pv. *oryzae*) and increased the yield of crop. Khabbaz *et al.*, (2003) reported that *P. fluorescens* strain pf1 and MMP were found to be most effective against *X. axonopodis* pv. *malvacearum* causing black arm of cotton.

2.2.3 Fungal Antagonists

The most exhaustively studied microorganism as a biocontrol agent is *Trichoderma* spp. The antagonistic potential of *Trichoderma* sp. was first demonstrated by Weindling (1932) on *Rhizoctonia solani*. Most of the studies remain centered on the use of antagonistic properties of *T. harzianum* (Sivan and Chet, 1982). Elad *et al.* (1983) observed that *T. harzianum* has antagonistic properties against *Sclerotium rolfsii*. *Pythium* spp., *Fusarium* spp, *R. solani* etc. Later on, other *Trichoderma* spp. and antagonistic fungi have been extensively studied and utilized in the biological control of many plant diseases (Chet and Inbar, 1994; Sen, 2000). *Trichoderma* spp. are effective against the quick wilt of pepper, rhizome rot of cardamom and ginger (KAU, 2002).

The inhibitory action of *Aspergillus niger*, *A. flavus* and *T. viride* on *R. solani* was demonstrated by Gokulapalan and Nair (1984). Gajbe and

Lanjewar (1991) observed that *A. niger* showed antagonism to *Macrophomina phaseolina, Phoma glomerata, Curvularia lunata, Dreschlera oryzae* and *Alternaria alternata. A. terreus, A. fumigatus* and *Penicillium citrinum* were found to be antagonistic under *in vitro* conditions to *M. phaseolina* (Mukherjee and Sen, 1992). Mandal *et al.* (1999) demonstrated the inhibitory effects of *Trichoderma pseudokoningii, T. hamatum, Talaromyces flavus* and *Trichothecium roseum* on the mycelial growth of wheat spot blotch pathogen, *Dreschlera sorokiniana.* In field conditions, application of *A. terreus* into press mud amended neutral soil showed highest efficacy in reducing sheath blight of rice caused by *R. solani* (Das and Roy, 2000). Singh and Singh (2000) observed maximum inhibition of the growth of *Alternaria solani* with the metabolites of *Aspergillus flavus* and *A. terreus* in laboratory tests.

The perusal of the literature revealed that not much work has been carried out linking a fungal antagonist and bacterial pathogen.

2.3 CHEMICAL ACTIVATORS INDUCING RESISTANCE AGAINST DISEASES

Among several disease control options available, the reigning management strategy adopted is the use of chemicals. Nearly all the compounds available are based on direct antibiotic principle but there is another group of chemicals which is based on development of resistance in the host.

Kúc *et al.* (1957) was the first to notice that D-phenyl alanine, D-alanine and DL-tryptophan injected into apple leaves increased resistance against scab without affecting the causal pathogen *in vitro.* Papavizas (1964) reported the high activity of β-amino butyric acid (BABA) against *Aphanomyces euteiches* causing root rot in peas. These were some of the earlier reports.

2.3.1 Salicylic Acid

White (1979) reported that exogenous application of salicylic acid to tobacco plants resulted in disease resistance which was correlated with PR gene expression. Salicylic acid induced resistance in sickle pod against the pathogen, *Alternaria cassiae* (Hoffland *et al.*, 1996). Kalix *et al* (1996) observed that salicylic acid could induce resistance in cucumber against *Cladosporium cucumerinum.* In rapeseed, resistance against *Pernospora* parasitica was elicited by salicylic acid (Narusaka *et al.*, 1999). Similarly, the role of salicylic acid in induction of resistance in tomato against *A. parasitica* and *A. solani* respectively has also been proven (Spletzer and Enyadi, 1999). Disease intensity of the disease caused by *Xanthomonas campestris* pv. *malvacearum* in cotton was reported to be lowest in Pseudomonas fluorescens and salicylic acid treated plants (Padmaja and Jayaraman, 2003).

2.3.2 Isonicotinic Acid (INA)

Metraux *et al.* (1991) discovered 2,6-dichloroisonicotinic acid (CGA 41396) and its methyl ester (CGA 41397; both referred to as INA) as agents able to induce systemic resistance in plants. Ensuing this discovery, several other reports poured in regarding the relevance of INA in induction of resistance. Uknës (1992) *et al.* reported that INA can induce resistance in arabidopsis against *Pernospora parasitica.* INA could also build up resistance in barely against *Erysiphe graminis* (Kogel *et al.*, 1994).

2.3.3 Benzothiadiazole (BTH)

A novel inducer, Benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester, CGA-245704 (BTH) was also widely exploited in the study of induced resistance. BTH has been reported to induce systemic acquired resistance (SAR) in tobacco against *Cercospora nicotianae, Eriwinia carotovora, Phytophthora parasitica, Pseudomonas syringe* and

TMV (Friedrich *et al.*, 1996). Gorlach *et al.* (1996) found that BTH systemically protected wheat against powdery mildew infection caused by *Erysiphe graminis* f.sp. *tritici.* Lawton *et al.* (1996) observed that BTH protected arabidopsis from *Pernospora parasitica, Pseudomonas syringe* and turnip wrinkle virus which demonstrates the versatility of the chemical. BTH could also induce systemic resistance in cucumber plants against *Pythium* damping off (Benhamou and Belanger, 1998).

2.3.4 Acibenzolar-S-Methyl (ASM)

Benzo (1 ,2 ,3)thiadiazole-7-carbothioate [acibenzolar-S-methyl (ASM)] was commercially marketed under the trade names Bion®, Actigard$^{(TM)}$ and Boost®. ASM has demonstrated efficacy in preventing or reducing diseases of plants caused by a variety of bacterial, fungal and viral pathogens (Friedrich *et al.*, 1996; Gorlach *et al.*, 1996; Kunz *et al.*, 1997). Gorlach *et al.* (1996) observed that ASM protected wheat against powdery mildew. Ishii *et al.* (1999) reported that ASM increased the resistance of Japanese pear to rust (*Gymnosporangium asiaticum*) and Scab (*Venturia nashicola*). Foliar application of ASM protected post harvest rock melons and Hami melons from fungal diseases (Huang *et al.*, 2000). Cowpea seedlings raised from ASM treated seeds inoculated at 7th day with *Collectotrichum destructum* displayed resistance (Akinwunmi *et al.*, 2001). Romero *et al.* (2001) observed that bell pepper plants sprayed with ASM showed resistance to subsequent infections with bacterial spot agent, *Xanthomonas axonopodis* pv. *vesicatoria.* Growth chamber experiments showed that ASM (300 μm) treatment protects pepper plants systemically and locally against *X. campestris* pv. *vesicatoria* (Buonaurio *et al.*,2002). The resistance inducing effect of ASM on apple against fireblight was demonstrated by Maxson-stein *et al.* (2002). Prats *et al.* (2002) observed that exogenous application of ASM induced resistance to rust infection in sunflower. Anith *et al.*(2004) reported that disease incidence

of actigard treated plants was significantly less than non-treated plants in tomato against *Ralstonia solanacearum*.

2.3.5 Ethephon

Basic pathogenesis related (PR) genes were highly inducible by treatment of plants with ethylene releasing compound, ethephon (Memelink *et al.*, 1990). Genes encoding acidic PR protein were moderately induced by ethephon treatment whereas the basic PR protein induction was maximum in ethephon treatment.

2.3.6 Potassium Phosphonate (Akomin-40)

Potassium phosphonate, a new formulation Akomin-40 (Rallis India Ltd.) indicated a maximum reduction of foliar infection (up to 86.40 per cent) four days after treatment whereas root-rot suppression (up to 70 per cent) eight days after treatment in pepper (Veena and Sarma, 2000). For the control of *Phytophthora* root rot, 0.3 per cent potassium phosphonate has been recommended for pepper (KAU, 2002). Root-rot caused by *Pythium* and *Phytophthora* in anthurium can be controlled by 0.3 per cent potassium phosphonate (KAU, 2002).

2.3.7 Inorganic Compounds

Gottstein and Kuc (1989) reported that solutions of K_3PO_4, K_2HPO_4, Na_3PO_4 and Na_2HPO_4 sprayed on the undersides of first and second true leaves of cucumber induced systemic resistance in leaves three and four to anthracnose caused by *Colletotrichum lagenarium*. It has been demonstrated that foliar spray of micronutrients on cucumber induced systemic protection against powdery mildew (Reuveni *et al.*, 1997).

2.4 MANAGEMENT OF BACTERIAL BLIGHT OF ANTHURIUM

There are only few works done in the area of management of bacterial blight of anthurium.

Knauss (1972) found that foliar sprays of 200-400 ppm streptomycin during summer months at an interval of four to seven days were ineffective against *Xanthomonas axonopodis* pv. *dieffenbachiae*. Application of streptocycline for the management of bacterial leaf blight pathogen of rice incited by *X. oryzae* pv. *oryzae* has been recommended (KAU, 2002).

Gangopadhyay (1998) reported that seed treatment with turmeric powder impregnated sodium bicarbonate in the proportion of 10: 1 at the rate of 1g kg^{-1} seed was found to control soil borne disease of rice. Foliar spray of the mixture at the rate of 1 g l^{-1} of water at maximum tillering stage of the crop reduced the incidence of rice diseases. Dhanya (2000) reported that turmeric and sodium bicarbonate mixture (10:1) at 0.15 per cent recorded the least percentage disease infection.

Mary *et al.* (1986) reported that a foliar spray of cowdung extract 20 g l^{-1} controlled bacterial blight of rice. Curative spraying with cowdung extract at the rate of 20 g l^{-1} was found very effective in reducing the disease incidence of bacterial blight affected paddy compared to Streptocycline and Bactrinol-100 at 500 ppm (Mary, 1996). Spraying 20 g l^{-1} of cowdung extract is recommended for the management of bacterial leaf blight of rice (KAU, 2002).

Hulloli *et al.* (1998) reported antibacterial property of certain neem products like plantolyte and agricare against *X. axonopodis* pv. *malvacearum*.

Materials and Methods

3. MATERIALS AND METHODS

3.1 ISOLATION OF THE PATHOGEN

The bacterial pathogen was initially isolated from infected anthurium samples (Plates 1a, 1b, 1c and 1d) collected from different parts of Thiruvananthapuram district including the Instructional farm, College of Agriculture, Vellayani. The affected parts were subjected to ooze test to find out the presence of bacterium. The infected portions with profuse ooze were selected, cut into bits and surface sterilized with 0.1 per cent $HgCl_2$ solution. These bits were then washed in three changes of sterile distilled water and placed in a drop of sterile distilled water on a sterile glass slide. The bits were then teased apart using sterilized blade and forceps and kept for one minute to allow the bacterial ooze to mix with water. The bacterial suspension thus obtained was streaked on Potato Sucrose Agar (PSA) medium to get well isolated colonies of the bacterium.

Composition of PSA medium

Potato	-	300.0 g
Na_2HPO_4	-	2.0 g
$CaNO_3$	-	0.5 g
Peptone	-	5.0 g
Sucrose	-	20.0 g
Agar agar	-	20.0 g
Distilled water	-	1000 ml
pH	-	6.8 – 7.0

The plates were incubated for 24 h at room temperature. Single colonies were selected on the basis of their colour, shape and slime

Plate 1a. Local symptoms on leaves of anthurium

Plate 1b. Water soaked lesions on the under surface of the leaves

Plate 1c. Systemic symptoms on the upper surface of the leaves

Plate 1d. Systemic symptoms on the lower surface of the leaves

Plate 1a.

Plate 1b.

Plate 1c.

Plate 1d.

production. The cultures were further purified by repeated streaking on PSA medium. The pure cultures thus obtained were then tested for pathogenicity and for proving Koch's postulate. For this a thick suspension of 24 h old growth of each isolate was prepared separately. The isolates were artificially inoculated on the leaves and at the collar region by giving pinpricks and rubbing with cotton dipped in bacterial suspension. The inoculated plants were then maintained under high humidity by giving frequent water sprays and covering with polythene bags to create favourable micro-climatic conditions to initiate infection.

When the artificially inoculated plants reproduced the symptoms of the disease, isolations were carried out from infected portion following the procedure described above. Single colonies thus obtained were compared with that of the original isolate. The culture that developed the symptoms on the host most rapidly was chosen and compared with the bacterial blight pathogen, *Xanthomonas axonopodis* pv. *dieffenbachiae* maintained in the KSCSTE project in the department of plant pathology. This culture of the pathogen was used for further studies.

3.2 ISOLATION OF ANTAGONISTS

Microbes antagonistic to bacterial blight were isolated from rhizosphere and phyllosphere of disease free anthurium plants grown in different parts of Thiruvananthapuram district.

3.2.1 Isolation of Microbial Antagonists from Rhizosphere

Microbial isolates were obtained from the rhizosphere by dilution plate technique (Waksman, 1992). One gram sample of the roots with adhering soil was washed with sterile distilled water and shaken in 250 ml Erlen Meyer flask containing 100 ml sterile distilled water at 150 rpm for 30 minutes. Serial dilutions were prepared up to the dilution 10^{-8}. One ml of the aliquot from the dilutions 10^{-4}, 10^{-6} and 10^{-8} were transferred to sterilized petridishes and plated with Martin's Rose Bengal Agar

(Martin, 1950), Soil Extract Agar (Allen, 1957), and Kuster's medium respectively and incubated at room temperature (27 ± 2°C) till colonies appeared. Typical colonies from each of these dilutions were transferred and pure cultures maintained in slants of appropriate medium.

3.2.2 *Isolation of* Microbial Antagonists from Phyllosphere

Disease free leaves were collected from healthy anthurium plants. The phyllosphere microflora were obtained by serial dilution and the pure cultures of the different colonies obtained were maintained in slants of appropriate medium for further studies.

3.3 SCREENING OF ANTAGONISTS

3.3.1 *In vitro* Screening of Bacterial Antagonists Against *Xanthomonas axonopodis* pv. *dieffenbachiae*

In this experiment apart from the local isolates, certain known bacterial isolates were also tested. *Pseudomonas putida* strain 89B61, *Bacillus subtilis* strain GBO3 and *B. pumilis* strain SE34 were kindly given by J.W.Kloepper, Auburn University, Alabama, USA. *P. fluorescens* strain PNO26R was procured from Department of Plant Pathology, College of Agriculture, Vellayani. The strain P1 of the same was isolated from the wettable powder formulation obtained from the Department of Plant Pathology, College of Agriculture, Vellayani.

Bacterial pathogen, *X. axonopodis* pv. *dieffenbachiae* was grown on PSA slants. One ml of sterilized distilled water was poured into each 24 h old PSA slants and properly shaken to get a uniform suspension (O.D. value-1). One ml of this suspension was added to 25 ml of the melted and cooled PSA medium contained in conical flasks, swirled and poured into each sterile petridish. Sterilized filter paper discs (1 cm diameter) dipped in the uniform suspension of 24 h old growth of each bacterial isolate was placed at the center of the bacterial pathogen seeded PSA medium. Three replications were maintained for each isolate. Petridishes without

inoculating the antagonists served as control. The inoculated plates were incubated at room temperature (27 ± 2°C). The inhibition zone produced surrounding the growth of bacterial antagonist on the lawn of *X. axonopodis* pv. *dieffenbachiae* was measured after 48h.

3.3.2 *In vitro* Screening of Fungal Antagonists *Against Xanthomonas axonopodis* pv. *dieffenbachiae*

X. axonopodis pv. *dieffenbachiae* was seeded in the media in sterile petrishes as described above. The fungal isolates were grown in Potato Dextrose Agar (PDA) medium in petriplates till they attained full growth in the petridish. A known isolate of *Trichoderma* (T_1) obtained from Department of Plant Pathology, College of Agriculture, Vellayani as wettable powder formulation was also used. Fungal discs of size 5 mm were cut from the growing end using a sterile cork borer and placed in the center of the bacteria seeded medium in plates. Three replications were maintained. Petridishes without inoculating the antagonists served as control. The inoculated plates were incubated at room temperature (27 ± 2°C). The inhibition zone produced surrounding the growth of fungal antagonist on the lawn of *X. axonopodis* pv. *dieffenbachiae* was measured after 48 h.

3.4 CHARACTERIZATION OF THE SELECTED ISOLATES

3.4.1 Characterization of the Bacterial Isolate, B_{16} Antagonistic to the Pathogen

Characterization and identification of the antagonist selected from the previous *in vitro* experiment was done according to the methods recommended in Manual of Microbiological Methods published by the Society of American Bacteriologists (SAB, 1957) and methods described in the Microbiology – A laboratory manual (Cappuccino and Sherman, 1992).

3.4.1.1 Morphological Character

Colony characters and cell morphology were studied from a 24 h old culture of the bacterium on solidified Nutrient Agar (NA) medium.

Composition

Peptone	:	10.0 g
Beef extract	:	5.0 g
Agar agar	:	20.0 g
Distilled water	:	1000 ml
pH	:	6.8

For studying the cell morphology; the bacterial cells were stained for gram reaction and observed under the oil immersion objective of a microscope. Endospore staining was also performed.

3.4.1.1.1 Growth of the Bacterial Isolate in Solid Medium

Abundance of growth, pigmentation, optical characteristics and form of bacterial growth were studied on NA slants. A straight line was drawn on NA slants using a loopful of the dilute bacterial suspension and incubated at room temperature. Observations were taken on the growth characteristics of the antagonist after 24 h.

Size, pigmentation, form, margin and elevation of the bacterial colonies were studied on NA media in petriplates. A loopful of dilute suspension of the isolate was streaked on NA plates and incubated at room temperature. Observations on growth characteristics were taken after 24 h.

3.4.1.1.2 Growth of the Isolate of the Bacterium in Liquid Medium

Nutrient broth was used for studying the growth of bacterial isolates in liquid medium. Five ml of sterilized broth was taken in a test tube and inoculated with a loopful of 24 h old growth of the bacterium. The distribution and appearance of growth was evaluated after 24 h.

3.4.1.1.3 Observations of Motility in Soft Agar Deeps

SIM agar was used for this purpose.

Composition

Peptone	:	30.0 g
Beef extract	:	3.0 g
Ferrous ammonium sulphate	:	0.2 g
Sodium thiosulphate:		0.025 g
Agar agar	:	3.0 g
Distilled water	:	1000 ml
pH	:	7.3

SIM agar deeps were inoculated with 24 h old culture of the bacterial isolate by stab inoculation. A control was maintained by stabbing the soft agar deeps with sterile needle loops. If microbe is actively mobile, turbidity radiates outward from stabbed line. Observations were recorded on the motility of the bacterium.

3.4.1.2 Physiological Characters

The isolate of the bacterium was tested for physiological characters. The tests were performed in triplicates and the observations were recorded in comparison with uninoculated control.

3.4.1.2.1 Oxygen Requirement

To determine whether the bacterium was aerobic or anerobic, nutrient agar (containing 0.005 per cent bromocresol purple) columns in test tube were inoculated by stabbing with the bacterial isolate using a straight inoculation needle. To create an anaerobic condition, agar surface was covered with sterile liquid paraffin oil to a depth of one centimeter in one set of tubes. The agar surface in the other set of tubes was kept open

without paraffin oil. Both sets of tubes were incubated at room temperature and observations were recorded. Yellowing of the medium from the top was indication of positive reaction.

3.4.1.2.2 Catalase Test

A loopful of 24 h old culture of the bacterial isolate was smeared on the glass slide and covered with a few drops of 20 vol. H_2O_2. The production of gas bubbles was indicative of catalase positive reaction.

3.4.1.2.3 Oxidase Test

The bacterial culture was grown on NA medium. Following the incubation period, a freshly prepared one per cent solution of tetramethyl-p-phenyl-diamine dihydrochloride solution was poured on to the plate so as to cover the surface and then decanted. The colonies of oxidase positive organism rapidly developed a purple colour.

3.4.1.2.4 Growth in 3-12 per cent NaCl

Peptone water with 0,3,6,9 and 12 percent NaCl were used for the test.

Peptone	:	1.0 g
NaCl	:	0, 3, 6, 9, 12 g
Distilled water	:	100 ml

The medium was dispensed in tubes, autoclaved and inoculated with 24 h old culture of the bacterium and incubated under laboratory conditions. Observation was recorded on the growth of the bacterium.

3.4.1.2.5 *Litmus* Milk Reaction

Litmus milk broth was used for this test.

Composition:

Skim milk powder	= 100.0 g
Litmus	= 0.075 g
Distilled water	= 1000 ml

The broth was dispensed in tubes and autoclaved at 121^0C at 15 lb pressure for 15 minutes. The sterile broth was then inoculated with 24 h old culture of the bacterium and incubated under laboratory conditions. Lactose fermentation will be indicated by change of colour of litmus from purple to pink whereas litmus reduction will be indicated by milk coloured litmus. Peptonization will be represented by litmus turning deep purple in upper portion of the tube, while the medium produces translucent, brown whey like appearance. Alkaline reaction on the other hand will be indicated by the colour of the medium remaining unchanged or changing to a deep blue colour. Observations were recorded accordingly.

3.4.1.2.6 Carbohydrate Fermentation

The broths used included Phenol red lactose, Phenol red dextrose and Phenol red sucrose broth.

Composition

Phenol red lactose	Phenol red dextrose	Phenol red sucrose
Trypticase = 10.0 g	Trypticase = 10.0 g	Trypticase = 10.0 g
Lactose = 5.0 g	Dextrose = 5.0 g	Sucrose = 5.0 g
NaCl = 5.0 g	NaCl = 5.0 g	NaCl = 5.0 g
Phenol red = 0.018 g	Phenol red = 0.018 g	Phenol red = 0.018 g

The medium was dispensed in tubes inoculated with 24 h old culture of the bacterium and incubated at 37°C. Phenol red was red at neutral pH, changed to yellow at acidic pH.

3.4.1.2.7 Hydrogen Sulphide Test

SIM Agar medium used for motility tests were used for this test also. The sterile soft agar deeps were stab inoculated with the bacterial culture and incubated for 24 – 48 h at 37°C. Production of H_2S was indicated by blackening along the stab.

3.4.1.2.8 Indole Production

Tryptone broth medium was used for the test.

Tryptone/casein digest	:	10.0 g
NaCl	:	5.0 g
Distilled water	:	1000 ml
pH	:	7.0

The medium was dispensed in tubes and autoclaved. Gnezda oxalic acid test strips were used for detecting indole production. Filter paper strips of size 5 x 50 mm were soaked in warm saturated solution of oxalic acid and cooled. When strips got covered with oxalic acid crystals, they were dried at room temperature and used without sterilizing.

The tryptone broth tubes were inoculated with the bacterium and oxalic acid strips were inserted into the tube by the side of the plug, incubated and observed regularly for 14 days. Change in colour of oxalic acid crystals on test strips to pink or red indicated indole production.

3.4.1.2.9 Methyl Red Test (MR test)

Methyl red peptone broth was used.

Composition

Proteose peptone	:	5.0 g
Glucose	:	5.0 g
K_2HPO_4	:	5.0 g
Distilled water	:	1000 ml
pH	:	7.0

The medium was dispensed in 5 ml aliquot in tubes and sterilized by steaming for 30 minutes for three successive days. Two sets of tubes

were inoculated with 24 h old culture of the bacterium for MR test. The tubes were incubated for seven days at room temperature.

A few drops of methyl red (0.1 g dissolved in 30 ml of 95 per cent ethyl alcohol and diluted to 50 ml with distilled water) was added to the culture tubes. A distinct red colour indicated positive MR reaction and an yellow colour with the methyl red indicator regarded as negative reaction.

3.4.1.2.10 Citrate Utilization Test

Simmon's citrate agar slants were used.

Composition

Ammonium dihydrogen phosphate	:	1.0 g
Dipotassium phosphate	:	1.0 g
NaCl	:	5.0 g
Sodium citrate	:	2.0 g
Magnesium sulphate	:	0.2 g
Agar	:	15 g
Bromothymol blue	:	0.08 g
Distilled water	:	1000 ml

The medium was dispensed in tubes and autoclaved. The culture of the bacterial isolate was streaked on the slants and incubated at 37°C. The positive reaction is indicated by the presence of growth.

3.4.1.2.11 Urease Test

Christensen's medium was used here.

Composition

Peptone	:	1.0 g
Glucose	:	1.0 g

NaCl	:	5.0 g
K_2HPO_4	:	2.0 g
0.2 per cent solution		
of phenol red	:	6 ml
Agar	:	20.0 g
Distilled water	:	1000 ml

Ninety ml aliquots of the medium were dispensed in 250 ml conical flasks and autoclaved. To each flask 10 ml of 20 per cent urea solution (sterilized by filtration) was added and dispensed in tubes in five ml quantities and slants were prepared. The slants were inoculated with test culture and observations were recorded for 15 days at regular intervals. Colour change of the medium from yellow to red was positive indication of urease activity.

3.4.1.2.12 Gelatin Liquefaction

Nutrient gelatin medium was used.

Composition

Peptone	:	10.0 g
Beef extract	:	5.0 g
Gelatin	:	120.0 g
Distilled water	:	1000 ml
pH	:	7.0

Gelatin was mixed together with all other ingredients and heated over a water bath until gelatin was dissolved. The medium was dispensed in test tubes to a depth of about five cm and sterilized at 10 lbs pressure for 20 minutes. The sterile condition of the medium was checked by observing it for two days. These gelatin columns were inoculated by stabbing with a straight inoculation needle charged with 24 h old culture

of the bacterium. The tubes were incubated and observed for liquefaction of gel column at regular intervals up to one month.

3.4.1.2.13 Starch Hydrolysis

The ability of the bacterium to hydrolyse starch was tested using starch medium containing 0.2 per cent soluble starch (Difco).

Composition

Peptone	:	10.0 g
Beef extract	:	5.0 g
Starch (Sol)	:	2.0 g
Agar	:	20.0 g
Distilled water	:	1000 ml
pH	:	7.0

Twenty four hour old bacterial culture was spot inoculated on the medium in plates. After four days of incubation, hydrolysis was tested by pouring Lugol's Iodine over the medium. A colourless/reddish brown zone around the bacterial growth in contrast to blue background of the medium was indicative of positive starch hydrolysis.

3.4.1.2.14 Lipid Hydrolysis

The medium of Sierra (1957) was used for this test.

Composition

Peptone	:	10.0 g
NaCl	:	5.0 g
$CaCl_2.H_2O$	:	0.1 g
Agar	:	20.0 g
Distilled water	:	1000 ml
pH	:	7.0

The medium was dispensed in 99 ml quantities in flasks autoclaved and cooled to 45°C. One ml of Tween-80 (oleic acid ester) was added to the medium and thoroughly mixed. The medium was poured in sterile petridishes and test bacterium was spot inoculated on the medium. The plates were incubated and observed at regular intervals for seven days. Opaque zone around the bacterial growth was indicative of positive lipid hydrolysis.

3.4.1.3 Studies on the Bacterial Antagonist, B_{16}

3.4.1.3.1 Growth of the Isolate on solid media

Nature of growth, colour, shape, extent of growth and type of margin of the isolate was studied on different solid media. A loopful of dilute suspension of the bacterium was streaked on different solid media in triplicate and kept for incubation at room temperature. Observations were recorded after 24 h.

The following media were used.

1. Nutrient Agar (NA)
2. Potato Sucrose Agar (PSA)
3. Potato Dextrose Agar (PDA)
4. King's B Agar (KB)
5. Yeast Extract Glucose Chalk Agar (YGCA)
6. Glucose Agar (GA)
7. Glucose Yeast Extract Agar (GYEA)

NA: Given above.

PSA : Given above

PDA

Potato	:	200.0 g
Dextrose	:	20.0 g

Agar	:	20.0 g
Distilled water	:	1000 ml
pH	:	6.8

KB

Peptone	:	20.0g
Dipotassium Hydrogen orthophosphate	:	1.5g
Magnesiumsulphate	:	1.5g
Glycerol	:	10ml
Distilled water	:	1000ml
pH	:	7.2

YGCA

Yeast extract	:	10.0 g
Glucose	:	10.0 g
Chalk ($CaCO_3$)	:	20.0 g
Distilled water	:	1000 ml
pH	:	7.2

GA

Beef extract	:	5.0 g
Peptone	:	3.0 g
Glucose	:	10.0 g
Agar	:	20.0 g
Distilled water	:	1000 ml
pH	:	6.8

GYEA

Yeast extract	:	5.0 g
Peptone	:	5.0 g
Glucose	:	10.0 g
Agar	:	20.0 g
Distilled water	:	1000 ml
pH	:	6.8

3.4.1.3.2 Familiarization with the Population Growth Dynamics of Bacterial Isolate

Peptone water was the medium used.

Composition

Peptone	:	1.0 g
Distilled water	:	100 ml

Five ml of the medium was dispensed in tubes and autoclaved. The sterile medium was inoculated with one ml of 24 h old bacterial suspension grown on nutrient broth using a sterilized pipette. The test was done in triplicate. Uninoculated medium was also maintained. Optical density of the medium was measured after 0, 30 min, 1 h, 2 h, 4 h, 6 h, 24h and 72 h using a spectrophotometer at 485 nm wavelength. Uninoculated medium was used as a blank.

3.4.1.3.3 Testing the Antagonist for Pathogenicity

A thick suspension of 24 h old culture of the isolate was prepared. The isolate was inoculated on leaves of anthurium by giving pinpricks and rubbing with cotton dipped in the bacterial suspension. The inoculated plants were then maintained under high humidity by giving frequent

sprays and covering with polythene bag to create favourable microclimatic conditions to initiate infection.

3.4.2 Characterization of the Fungal Antagonist, F_8

For ascertaining the identity of the fungal antagonist, the pure culture of the same was sent to Agharkar Research Institute, Pune.

3.4.2.1 Growth in PDA

To determine the growth rate of the fungus, the selected isolate was grown on PDA. Fungal discs of 5 mm were cut using a sterile cork borer and placed aseptically at the center of the medium in sterile petriplates. Three replications were maintained. The inoculated plates were incubated at room temperature (27 ± 2°C) till the fungus grew and covered the entire medium in the petriplate. Growth rate was determined by expressing the radial growth per minute. Other characters of the culture such as colour, sporulation etc. were also noted.

3.5 SCREENING OF CHEMICAL ACTIVATORS

3.5.1 *In vitro* testing of Chemical Activators for their Antibacterial Effects Against *X. axonopodis* pv. *dieffenbachiae*

The *in vitro* sensitivity of the bacterium to six different chemical activators at three different doses was tested. The following were the treatments used for study.

1. Potassium phosphonate (Akomin)

1.1 100ppm

1.2 300ppm

1.3 500ppm

2. Acibenzolar-s-methyl (ASM) (Actigard 50)

2.1 10ppm

2.2 25ppm

2.3 50ppm

3. Salicylic Acid (SA)

3.1 1mM

3.2 5mM

3.3 10mM

4. Dipotassium hydrogen phosphate (K_2HPO_4)

4.1 25mM

4.2 50mM

4.3 100mM

5. Ethephon (Ethrel)

5.1 25ppm

5.2 50ppm

5.3 100ppm

6. Ferric chloride ($FeCl_3$)

6.1 1mM

6.2 5mM

6.3 10mM

Actigard ™ 50WG containing active ingredient ASM manufactured by Novartis Crop Protection Inc., Greensboro, NC was kindly provided by T.M.Momol, University of Florida, USA.

Solutions of these chemical activators of above mentioned concentrations were prepared. Sterile filter paper discs of 10 mm diameter were dipped in the appropriate solutions and placed over PSA medium seeded with 48-h-old culture of *X. axonopodis* pv. *dieffenbachiae*. The treatments were replicated thrice. A control plate was also maintained by dipping sterile filter paper discs in sterile water and placing them over bacteria seeded PSA medium. Observations on the zone of inhibition were recorded after 48 h.

3.5.2 *In vivo* Testing of Chemical Activators

In order to assess the efficacy of these chemical activators against bacterial blight disease under *in vivo* conditions, a pot culture experiment was laid out in the glass house of Department of Plant Pathology in Completely Randomized Design with 19 treatments and three replications. Cancan, a hybrid variety of anthurium, highly susceptible to bacterial blight disease was selected for the study. The following 18 treatments *viz.*, potassium phosphonate (100, 300 and 500ppm), ASM (10, 25 and 50ppm), salicylic acid (1mM, 5mM and 10mM) dipotassium hydrogen phosphate (25, 50and 100mM), ethephon (25, 50 and 100ppm) and ferric chloride (1, 5 and 10 mM) were given seven days before artificial inoculation of the pathogen. Six month old tissue culture anthurium plants were artificially inoculated with 24 h old *X. axonopodis* pv.*dieffenbachiae* by giving pinpricks at the collar region and rubbing with cotton dipped in the bacterial suspension. The inoculated plants were covered with polythene bags and high humidity was maintained till the initiation of disease symptoms. A control plant was also maintained without giving any treatment prior to inoculation of the pathogen.

Scoring of the disease was done based on the disease score chart developed by Dhanya (2000) (Table 1). The observations were recorded on per cent disease infection.

Table 1. Descriptive keys for scoring bacterial blight of anthurium

Score	Percentage of infection	Description of disease development
0	0	No infection
1	1 – 5	Lesion at pinpricks
2	6 – 25	Lesion at pinpricks along with yellowing of 1 – 2 leaves
3	26 – 50	Lesion of size 1.2 x 0.5 cm along with yellowing of 1-2 leaves
4	51 – 75	Yellowing of all the leaves with blackening of petioles
5	76 – 100	Complete death

3.5.2.1 Biochemical Studies

Leaf samples of the different treatments were collected for estimating changes in the activity of phenols and defense related enzymes such as phenyl alanine ammonia lyase (PAL), peroxidases (PO) and polyphenyl oxidase (PPO). Leaf samples were taken after inoculation with the pathogen.

3.5.2.1.1 Total Phenol

Total phenol content was estimated following the procedure described by Bray and Thorpe (1954).

One gram of leaf sample was ground in 10 ml of 80 per cent ethanol. The homogenate was centrifuged at 10,000 rpm for 20 min, supernatant was saved and residue was extracted with five times the volume of 80 per cent ethanol and centrifuged. The supernatant was evaporated to dryness and the residue was dissolved in a known volume of distilled water (5 ml). An aliquot of 0.3 ml was pipetted out and made upto 3 ml with distilled water. Folin-ciocalteau reagent (0.5 µl) was added and 2.0 µl of 20 per cent sodium carbonate solution was added to each tube after three min. This was mixed thoroughly and kept in boiling water for one min. This was cooled and absorbance was measured at 650

nm against reagent blank. Standard curve was prepared using different concentrations of catechol and expressed in catechol equivalents and µg per g leaf tissue on fresh weight basis.

3.5.2.1.2 Phenyl Alanine Ammonia Lyase (PAL)

PAL activity was analysed using the procedure described by Dickerson *et al.* (1984).

The enzyme extract was prepared by homogenizing one g leaf sample in 5 ml of 0.1 M sodium borate buffer (pH 8.7) containing 0.05 g of poly vinyl pyrrolidone using chilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant was used for the assay. The reaction mixture contained 3 ml of 0.1 M sodium borate buffer, 0.2 ml enzyme extract. The reaction mixture and blank were incubated at 40°C for 30 min and reaction was stopped by adding 0.2 ml of 3 N hydrochloric acid. The absorbance was read at 290 nm in spectrophotometer.

PAL activity was expressed as µg of cinnamic acid produced per min per g on fresh weight basis.

3.5.2.1.3 Peroxide (PO)

Peroxidase activity was determined according to procedure described by Srivastava (1987).

Leaf samples of 200 mg were homogenized in 10 ml of 0.1 M sodium phosphate buffer (pH 6.5) to which 0.05 g of poly vinyl pyrrolidone was added. Homogenization was done at 4°C using pestle and mortar. The homogenate was filtered through a muslin cloth and centrifuged at 3,000 rpm for 15 min at 4°C. The supernatant was used as enzyme extract. The reaction mixture consisting of 1 ml of 0.04 M pyrogallol and 50 µl enzyme extract was taken in both reference and sample cuvettes mixed and placed in the spectrophotometer with reading adjusted to zero at 420 nm. The enzyme reaction was started by adding

one ml of one per cent hydrogen peroxide into sample cuvettes and change in absorbance was measured at 30 s interval.

3.5.2.1.4 Polyphenol Oxidase (PPO)

PPO was determined as per the procedure given by Mayer *et al.* (1965).

Leaf samples of 200 mg were homogenized in one ml of 0.1 M sodium phosphate buffer (pH 6.5) to which 0.05 g poly vinyl pyrrolidone was added. Homogenization was done at 4°C using pestle and mortar. The homogenate was filtered through a muslin cloth and centrifuged at 5000 rpm for 15 min at 4°C and the supernatant was used as enzyme extract. The reaction mixture contained one ml of 0.1 M sodium phosphate buffer and 1.0 ml of 0.01 M catechol. Cuvettes were placed in spectrophotometer and absorbance was recorded at 495 nm and PPO activity expressed as change in absorbance of reaction mixture per ml per g on fresh weight basis.

3.6 MANAGEMENT OF BACTERIAL BLIGHT OF ANTHURIUM

The selected treatments from previous experiments were tested for their effects under *in vivo* condition. For this a pot culture experiment was laid out in the glass house of the Department of Plant Pathology in a Completely Randomized Block Design with eight treatments and three replications. Ten month old tissue culture anthurium plants of variety Cancan was chosen for the experiment. The treatments included the two best antagonistic organisms (a bacteria and a fungus), two best chemical activators, crude extract of neem cake, 0.15 per cent of turmeric powder + sodium bicarbonate (10 : 1), streptocycline at 100 ppm and control. The treatments were given as root dip and as foliar spray prior to two weeks and one week of pathogen inoculation respectively. Plants were inoculated with *X. axonopodis* pv. *dieffenbachiae* by artificial inoculation as in the previous *in vivo* experiment.

The disease severity was scored at fortnightly intervals until 2 months using the score chart and per cent disease intensity was worked out as in previous *in vivo* experiment.

3.7 STATISTICAL ANALYSIS

The data generated from the experiment were subjected to analysis of variance (ANOVA) after appropriate transformations wherever needed.

Results

4. RESULTS

4.1 ISOLATION OF THE PATHOGEN

Seven bacterial isolates were collected from Thiruvananthapuram district and named from Xad 1 to Xad 7. These isolates were artificially inoculated on the leaves as well as collar region of *Anthurium andreanum* var. Cancan. The isolate (Plate 2) which reproduced the original symptoms rapidly within a period of one week was selected as the isolate which was used for further studies. The most virulent isolate was Xad 6.

4.2 ISOLATION OF ANTAGONISTS

Seventeen isolates of the bacteria and eight isolates of fungi were obtained from samples collected from Vellayani, Poojappura, Kowdiar and Thiruvallam.

4.2.1 Isolation of Microbial Antagonists from Rhizosphere

Eleven bacterial isolates and five distinct fungal isolates were obtained from the rhizosphere. Bacterial colonies were obtained at 10^{-6} dilutions in soil extract agar whereas the fungal colonies developed in Martin's Rose Bengal agar medium in 10^{-4} dilutions. No actinomycete colonies were obtained.

4.2.2 Isolation of Microbial Antagonists from Phyllosphere

Phyllosphere contributed six bacterial isolates and three fungal isolates. Colonies of bacteria and fungi were obtained from 10^{-6} and 10^{-4} dilutions in soil extract agar and Martin's Rose Bengal agar medium respectively. Actinomycete colonies were not obtained from phyllosphere.

Plate 2. Xad 6 (*Xanthomonas axonopodis* pv. *dieffenbachiae*)

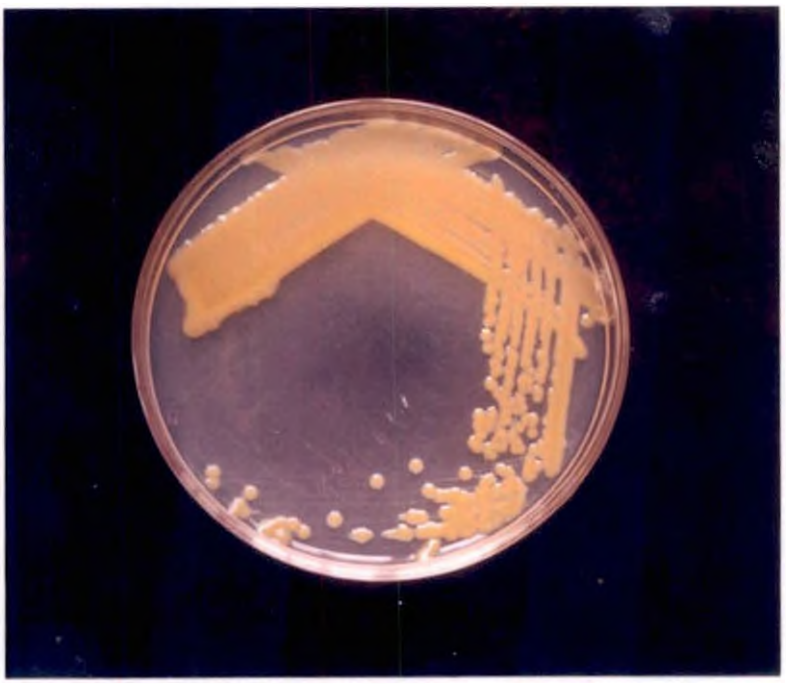


Plate 2.

4.3 SCREENING OF ANTAGONISTS

4.3.1 *In vitro* Screening of Bacterial Antagonists Against *Xanthomonas axonopodis* pv. *dieffenbachiae*

Seventeen isolates of bacteria obtained from serial dilution of rhizosphere and phyllosphere of anthurium plants were numbered from B_1 to B_{17}. Five known bacterial antagonists *viz.*, SE34, PNO26R, GBO3, 89B61 and P1 were also screened. They were screened for antagonism against *X. axonopodis* pv.*dieffenbachiae* and the inhibition zones around the filter paper disc dipped in antagonists were measured and expressed in mm. The inhibition zones exhibited by different bacterial isolates are presented in Table 2.

Among the seventeen bacterial isolates, maximum inhibition zone of 16.39 mm was exhibited by the isolate B_{16} (Plate 3a). This was followed by isolates B_{12} and B_9, their zones of inhibition being 8.26 and 8.06 mm respectively (Plates 3b and 3c). The isolates B_5 and B_{17} exhibited inhibition of the pathogen up to 7.75 and 7.73 mm respectively (Plates 3d and 3e). The inhibition zone of GBO3, P1 and B_1 were 6.26, 5.33 and 5.06 mm respectively. The isolates SE34, B_{10} and B_4 developed an inhibition zone of 4.48, 4.15 and 2.86 mm respectively. The isolates 89B61, PNO26R, B_{15}, B_{14}, B_{13}, B_{11}, B_8, B_7, B_6, B_3 and B_2 did not inhibit the growth of the pathogen.

The isolate B_{16} was selected as the best isolate as it not only had the highest inhibition zone but also the zone was developed after 24 h itself while the others took another 24 h to produce a well defined inhibition zone. The isolate B_{16} was subjected to characterization studies for identification. All further studies were done on this isolate.

4.3.2 *In vitro* Screening of Fungal Antagonists *Against Xanthomonas axonopodis* pv. *dieffenbachiae*

Rhizosphere and phyllosphere of anthurium plants contributed nine isolates of fungi. A known isolate of *Trichoderma* (T_1) available as

Table 2 *In vitro* growth inhibition of *Xanthomonas axonopodis* pv. *dieffenbachiae* by bacterial isolates

Sl. No.	Isolates	*Inhibition zone in mm
1	B_1	5.06 (2.46)
2	B_2	0 (1)
3	B_3	0 (1)
4	B_4	2.86 (1.96)
5	B_5	7.75 (2.96)
6	B_6	0 (1)
7	B_7	0 (1)
8	B_8	0 (1)
9	B_9	8.06 (3.01)
10	B_{10}	4.15 (2.27)
11	B_{11}	0. (1)
12	B_{12}	8.26 (3.04)
13	B_{13}	0 (1)
14	B_{14}	0 (1)
15	B_{15}	0 (1)
16	B_{16}	16.39 (4.17)
17	B_{17}	7.73 (2.95)
18	SE34	4.48 (2.34)
19	PNO26R	0 (1)
20	GB03	6.26 (2.69)
21	89B61	0 (1)
22	P1	5.33 (2.52)
23	Control	0 (1)

* Average of three replications

Figures in parenthesis are $\sqrt{x + 1}$ value

CD = 0.00946

Plate 3a. Antagonism exhibited by B_{16} on *Xanthomonas axonopodis* pv. *dieffenbachiae*

Plate 3b. Antagonism exhibited by B_{12} on *Xanthomonas axonopodis* pv. *dieffenbachiae*

Plate 3c. Antagonism exhibited by B_9 on *Xanthomonas axonopodis* pv. *dieffenbachiae*

Plate 3d. Antagonism exhibited by B_5 on *Xanthomonas axonopodis* pv. *dieffenbachiae*

Plate 3e. Antagonism exhibited by B_{17} on *Xanthomonas axonopodis* pv. *dieffenbachiae*

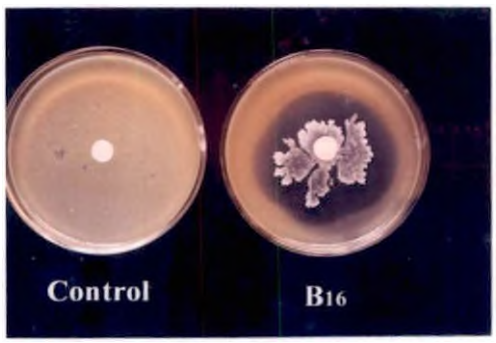


Plate 3a.

Plate 3b.

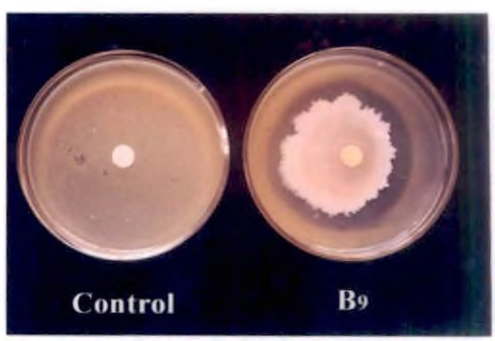


Plate 3c.

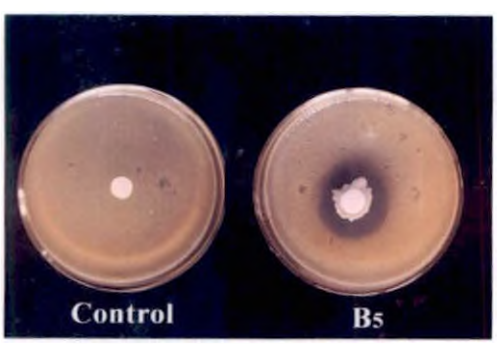


Plate 3d.

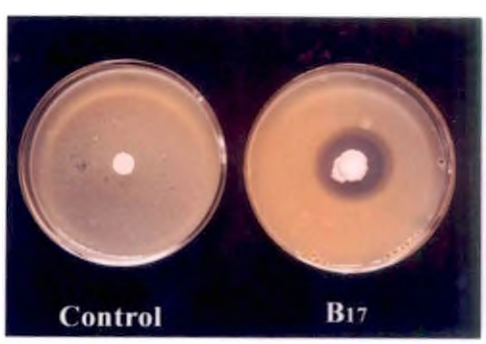


Plate 3e.

wettable powder from the Department of Plant Pathology, College of Agriculture, Vellayani was also included in the study. Each of them was tested for antagonism against the pathogen, *X. axonopodis* pv. *dieffenbachiae*. The inhibition zones measured are presented in Table 3.

The statistical analysis of the data presented in the table indicates that among the ten fungal isolates the inhibition zone produced by six isolates were significantly superior to control. The isolate F_8 produced the maximum inhibition zone of 10.66 mm (Plate 4). This was followed by the isolate F_9, the inhibition zone of which was only 3.46 mm. The isolates,F_4 and F_1 having an inhibition zone of 2.55 and 2.40 mm respectively were on par with each other. Similarly the isolates F_1 and F_2 were also on par with each other. The isolate F_{10} produced the lowest inhibition zone of 0.63 mm. The isolates T_1, F3, F_5, F_6 and F_7 did not exhibit any antagonism against *X. axonopodis* pv. *dieffenbachiae*.

The isolate F_8 was selected for further studies as it was the isolate which inhibited the pathogen the most. This isolate was further used in the *in vivo* experiment.

4.4 CHARACTERIZATION OF THE SELECTED ISOLATES

4.4.1 Characterization of the Bacterial Isolate, B_{16} Antagonistic to the Pathogen

4.4.1.1 Morphological Characters

The bacterium was a gram positive rod. They also produced endospores.

4.4.1.1.1 Growth of the Bacterial Isolate in Solid Medium

In nutrient agar slants there was abundant growth which was white in colour and opaque. There was a continuous thread like growth with irregular edges.

In nutrient agar plates, the isolate produced white, small colonies which were circular with more or less defined margin.

Table 3 *In vitro* growth inhibition of *Xanthomonas axonopodis* pv. *dieffenbachiae* by fungal isolates

Sl No.	Isolates	*Inhibition zone in mm
1	F_1	2.40 (1.84)
2	F_2	1.68 (1.64)
3	F_3	0 (1)
4	F_4	2.55 (1.88)
5	F_5	0 (1)
6	F_6	0 (1)
7	F_7	0 (1)
8	F_8	10.66 (3.42)
9	F_9	3.46 (2.11)
10	F_{10}	0.63 (1.28)
11	T_1	0 (1)
12	Control	0 (1)

* Average of three replications

Values in parenthesis are $\sqrt{x+1}$ value

CD =0.202

Plate 4. Antagonism exhibited by *Talaromyces* sp. (F_8)

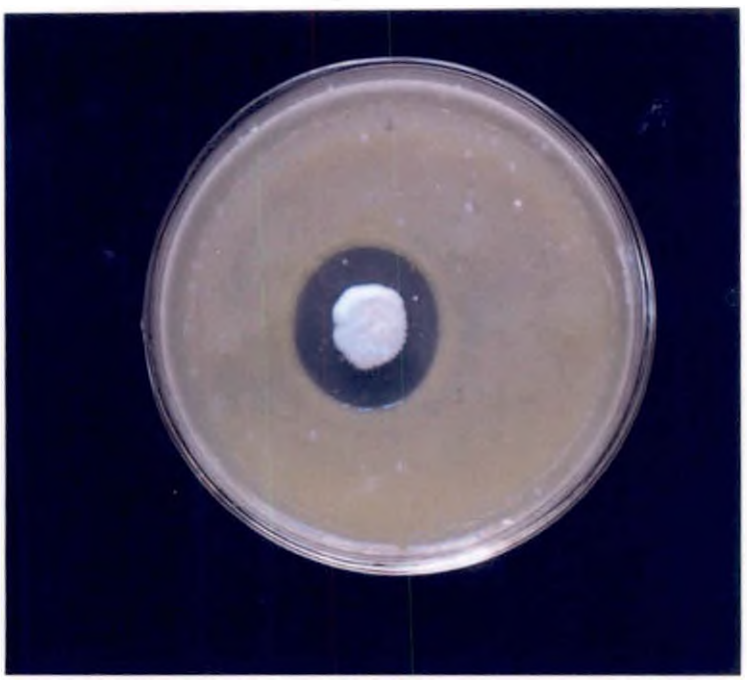


Plate 4.

4.4.1.1.2 Growth of the Bacterial Isolate in Liquid Medium

The bacterial isolate produced pellicle type of growth which was indicated by thick pad like growth on the surface.

4.4.1.1.3 Observations of Motility in Soft Agar Deeps

The isolate was non-motile since the bacterial growth was restricted to the stab alone and was not seen radiating from the stabbed line.

4.4.1.2 Physiological Characters

4.4.1.2.1 Oxygen Requirement

The bacterial isolate was aerobic since the growth and change of blue colour of the medium containing 0.005per cent bromocresol purple to yellow was observed only in the tube without paraffin sealing.

4.4.1.2.2 Catalase Test

Catalase positive reaction was shown by the isolate.

4.4.1.2.3 Oxidase Test

As the colonies of the isolate did not develop a purple colour on addition of freshly prepared 1 per cent solution of tetramethyl-p-phenyl-diamine dihydrochloride solution, the isolate tested was negative to the test.

4.4.1.2.4 Growth in 3-12 per cent NaCl

There was bacterial growth in all the tubes with NaCl of concentrations 0, 3, 6, 9 and 12 per cent, indicating their tolerance level of NaCl.

4.4.1.2.5 Litmus Milk Reaction

The test indicated peptonization which was represented by change in colour of litmus to deep purple in upper portion while the medium turned translucent with brown whey like appearance.

4.4.1.2.6 Carbohydrate Fermentation

In the tubes containing the sugars, dextrose and sucrose, the reaction was acidic. In the case of lactose, the reaction was neutral.

4.4.1.2.7 Hydrogen Sulphide Test

The isolate did not produce any hydrogen sulphide as no blackening was seen along the stab.

4.4.1.2.8 Indole Production

The oxalic acid crystals on the test strip did not turn pink or red, which indicated the absence of indole production by the bacterial isolate.

4.4.1.2.9 Methyl Red Test

The isolate gave negative MR test as indicated by the development of yellow colour in the culture tube when a few drops of methyl red solution were added.

4.4.1.2.10 Citrate Utilization Test

The isolate did not grow on the Simmon's citrate agar slants indicating negative reaction.

4.4.1.2.11 Urease Test

The isolate gave negative urease test since there was no change in colour of the medium from yellow to red.

4.4.1.2.12 Gelatin Liquefaction

The isolate liquefied gelatin in the medium

4.4.1.2.13 Starch hydrolysis

The isolate hydrolyzed starch since a colourless or reddish zone was observed around the bacterial growth in contrast with the blue background of the medium.

4.4.2.14 Lipid Hydrolysis

An opaque zone was observed around the bacterial growth indicating hydrolysis of lipid.

The properties of the isolate B_{16} derived from the results of critical physiological and biochemical tests conducted and summarized in table 4 were compared with the characters of *Bacillus*. From the results of the above tests isolate B_{16} has been tentatively classified under the genus *Bacillus*.

4.4.1.3 Studies on the Selected Bacterial Isolate, B_{16}

4.4.1.3.1 Growth of the Isolate on Solid Media

The growth of the bacterium on seven different solid media was tested and the results are presented in table 5.

On the seven solid media tested, abundant growth was observed in nutrient agar (Plate 5). Apart from being abundant, the growth was also discernible. Moderate growth was observed in PSA, PDA and KB. On YGCA, GA and GYEA the growth was scanty.

4.4.1.3.2 Familiarization with the Population Growth Dynamics of Bacterial Isolate

The growth dynamics was studied on peptone water. The data on optical density of the broth culture measured after 0, 30 min, 1, 2, 4, 6, 24, 48 and 72 h are given in table 6 and fig 1. From the graph it can be seen that there was a steady increase in growth from 0 to 1h. From 1 h to 4 h the growth remained constant *i.e.*, the OD value was 0.220. In the sixth hour there was a drop in the growth *i.e.*, the OD value touched down to 0.197.The decline continued slowly and after 72 h the OD value was 0.169.

Table 4 Biochemical characters of B_{16} (*Bacillus* sp.)

Sl No.	Biochemical tests	Results
1	Oxygen requirement	+
2	Catalase test	+
3	Oxidase test	-
4	Growth in 3-12 per cent NaCl	+
5	Litmus milk reaction	Peptonization
6	Carbohydrate fermentation Dextrose Sucrose Lactose	 Acidic Acidic Neutral
7	Hydrogen sulphide test	-
8	Indole production test	-
9	MR test	-
10	Citrate utilization test	-
11	Urease test	-
12	Gelatin liquefaction	+
13	Starch hydrolysis	+
14	Lipid hydrolysis	+

Table 5 Growth of the isolate B_{16} (*Bacillus* sp.) on different solid media

Sl.No.	Medium	Nature of colony and colour	Growth
1	NA	Distinct white convex colonies	+++
2	PSA	Off white raised colonies with irregular margin	++
3	PDA	Off white raised colonies with irregular margin	++
4	KB	Off white raised colonies with irregular margin	++
5	YGCA	Dirty white flat colonies	+
6	GA	Dirty white flat colonies	+
7	GYEA	Dirty white flat colonies	+

+++ Good

++ Moderate

+ Scanty

Plate 5. Growth of *Bacillus* sp. (B_{16}) on nutrient agar medium

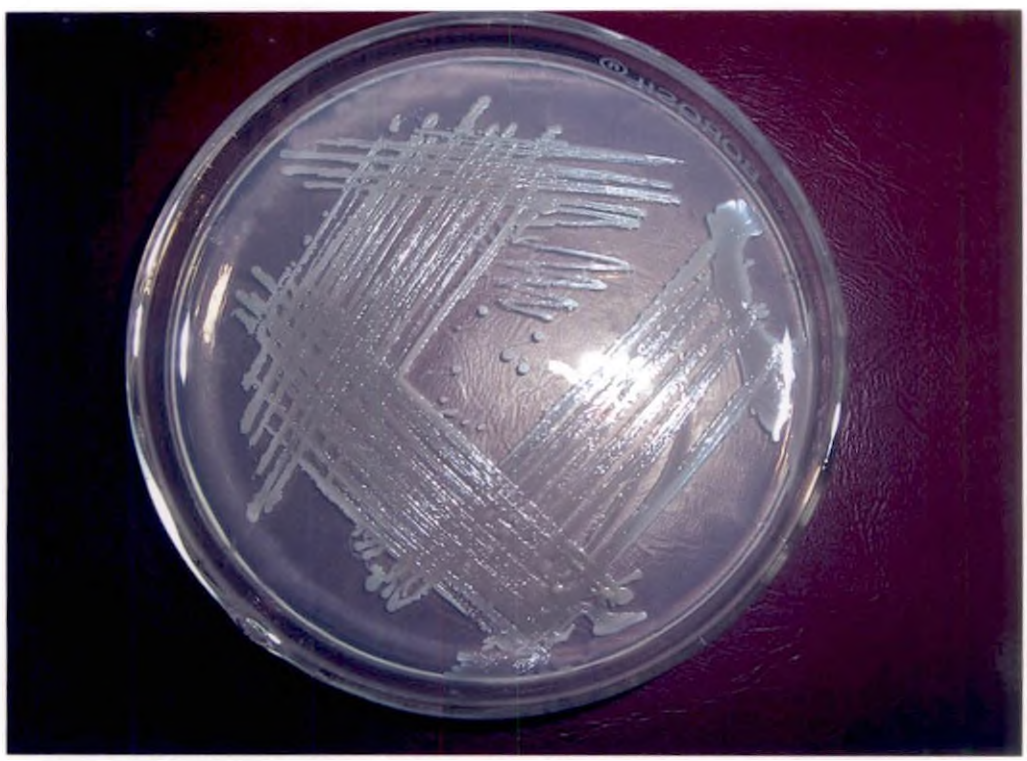


Plate 5.

Table 6 Optical density (OD) value of B_{16} (*Bacillus* sp.) at 485 nm

Incubation period	OD value at 485 nm
0	0.056
30 min	0.112
1h	0.220
2h	0.220
4h	0.220
6h	0.197
24h	0.185
48h	0.172
72h	0.169

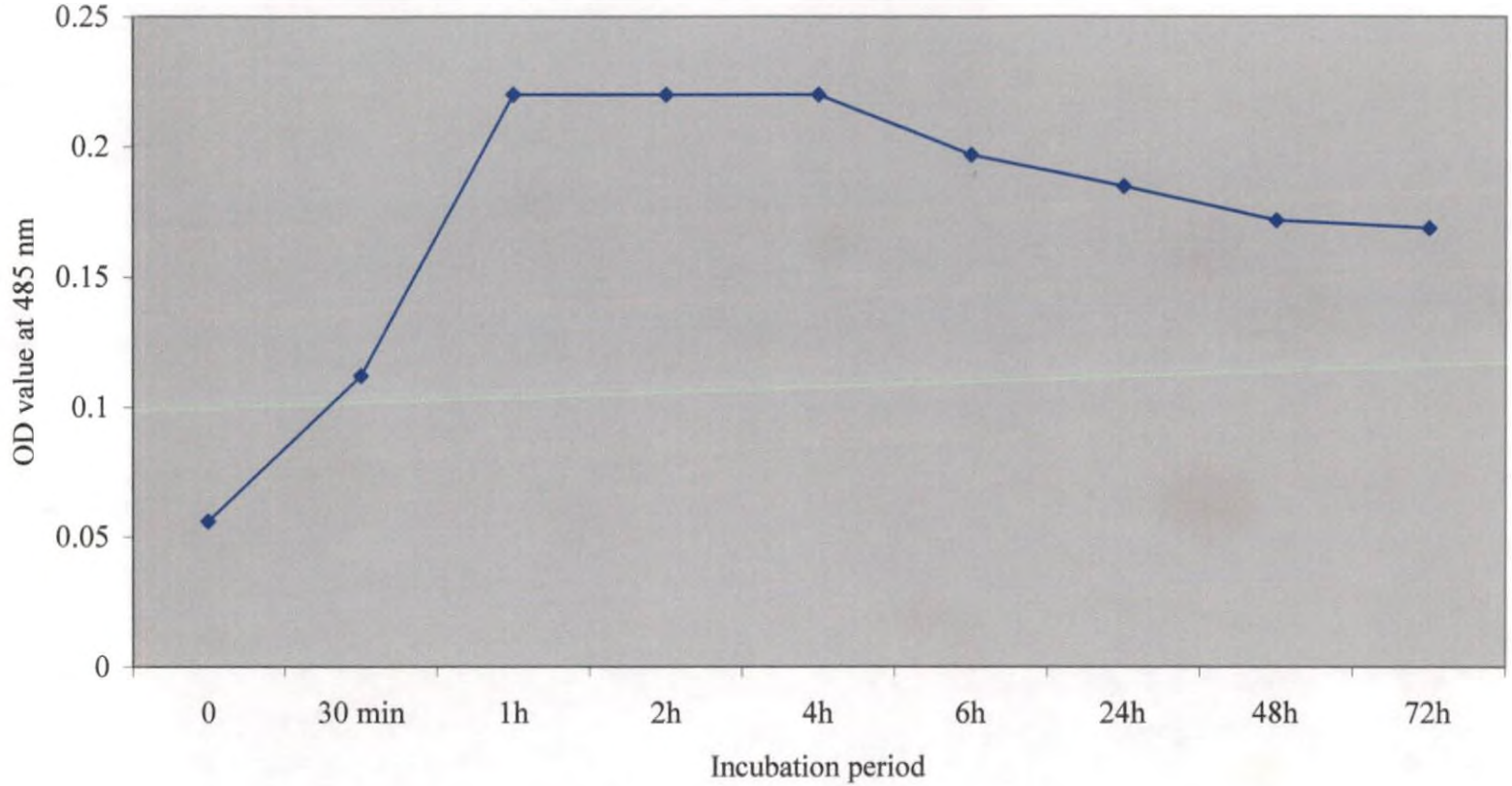


Fig. 1. Optical density (OD) value of B16 *(Bacillus* sp.) at 485 nm

4.4.1.3.3 Testing the Bacterial Isolate for Pathogenicity

Following inoculation of the isolate on anthurium, no symptom developed on the plants. This showed that the isolate was not pathogenic.

4.4.2 Characterization of the Fungal Antagonist,F_8

The fungus was identified as *Talaromyces* sp. (close to *Talaromyces gossypi*) by Agharkar research institute, Pune.

4.4.2.1 Growth in PDA

The fungus *Talaromyces* sp. attained full growth in the petriplate after nine days in all the three replications. The growth rate was 0.42mm h^{-1}. Initially the fungal colonies appeared with white fluffy growth. On initiation of sporulation, the fluffiness was replaced by yellow and resin coloured spores.

4.5 SCREENING OF CHEMICAL ACTIVATORS

4.5.1 *In vitro* Testing of Chemical Activators for their Antibacterial Effects Against *X. axonopodis* pv. *dieffenbachiae*

Six different chemical activators viz., potassium phosphonate, acibenzolar-s-methyl (ASM), salicylic acid (SA), dipotassium hydrogen phosphate (K_2HPO_4), ethephon and ferric chloride ($FeCl_3$) at three different doses were examined for any possible antibacterial effects on the pathogen. None of the chemicals at any of their doses inhibited the bacteria, conforming to one of the characters of an activator.

4.5.2 *In vivo* Testing of Chemical Activators

Anthurium andreanum var Cancan highly susceptible to bacterial blight was used for the study. Six month old tissue culture plants were artificially inoculated with the bacterial blight pathogen, *X. axonopodis* pv. *dieffenbachiae* one week after applying the chemical activators. Observations on per cent infection of bacterial blight were recorded three

weeks after inoculation. The percentage infection of the disease is presented in table 7 and Fig. 2.

All the plants developed bacterial blight infection ranging from 12.93 per cent to as high as 90.31 per cent. The results revealed that among the treatments, 500ppm potassium phosphonate was the best followed by ASM at 25 and 50ppm which were statistically on par with each other and significantly superior to all the other treatments in reducing the per cent intensity of bacterial blight of anthurium; their intensity being 12.93, 14.77 and 16.19 and disease reduction over control being 85.15, 83.04 and 81.41 per cent respectively. In the case of all treatments except ASM, as the concentration of the chemical increased there was a corresponding increase in its effect on bacterial blight disease management. Though all the treatments were superior to control, 500ppm potassium phosphonate, ASM at 25 and 50ppm were the best treatments which reduced bacterial blight intensity below 16.19 per cent.

Among the doses of potassium phosphonate, the highest dose of 500ppm was significantly superior to 300ppm and 100ppm with the per cent disease intensity 43.65 and 74.40 respectively. Between the lower doses (300 and 100ppm) of potassium phosphonate also the higher dose (300ppm) recorded statistically superior disease reduction over its lower dose (100ppm). The lowest concentration (100ppm) of potassium phosphonate was third in terms of effectiveness (74.40 per cent disease intensity).

Among the different concentrations of ASM, the lowest dose of 10ppm recorded high disease intensity (51.31 per cent). The other two doses 25ppm and 50ppm were statistically on par and significantly superior to their lowest dose of 10ppm in reducing the per cent disease intensity.

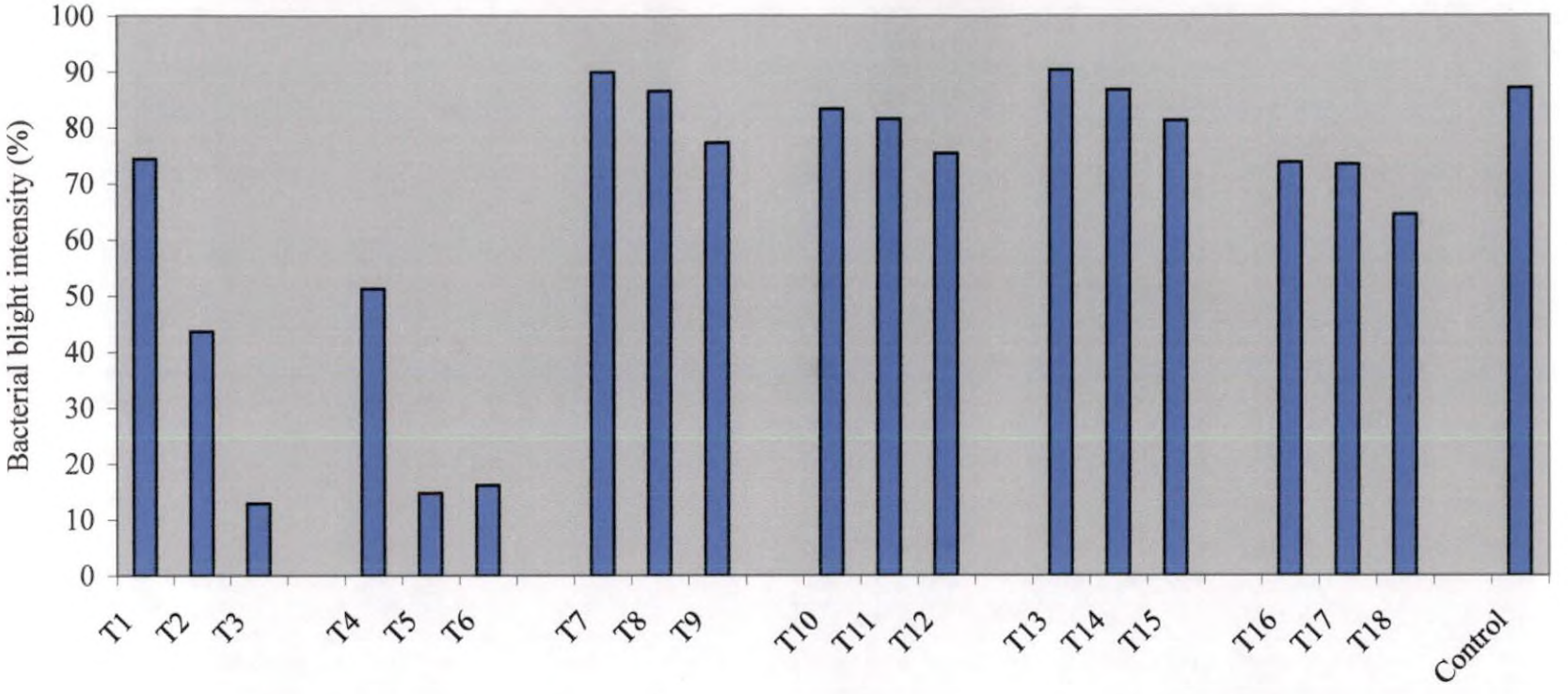
Table 7 Effect of chemical activators on bacterial blight of anthurium

Sl No.	Treatments	Bacterial blight intensity (%)	Per cent disease reduction over control
1	100 ppm Potassium phosphonate	74.40 (59.58)	14.55
2	300 ppm Potassium phosphonate	43.65(41.33)	49.87
3	500 ppm Potassium phosphonate	12.93(21.07)	85.15
4	10ppm ASM	51.31(45.73)	41.07
5	25ppm ASM	14.77(22.59)	83.04
6	50ppm ASM	16.19(23.72)	81.41
7	1mM SA	89.93(71.47)	-3.28
8	5mM SA	86.60 (68.50)	5.39
9	10mM SA	77.28 (61.51)	11.24
10	25mM K_2HPO_4	83.31 (65.86)	4.32
11	50mM K_2HPO_4	81.60 (64.57)	6.28
12	100mM K_2HPO_4	75.44(60.27)	13.36
13	25ppm Ethephon	90.31 (71.84)	-3.72
14	50ppm Ethephon	86.75 (68.63)	0.37
15	100ppm Ethephon	81.25 (64.32)	6.68
16	1mM $FeCl_3$	73.77 (59.17)	15.26
17	5mM $FeCl_3$	73.45 (58.96)	15.64
18	10mM $FeCl_3$	64.51 (53.41)	25.91
19	Control	87.07 (68.91)	0

Average of three replications

Figures in parenthesis are transformed values (angular transformation)

CD=3.55



Treatments

T_1	100 ppm Potassium phosphonate	T_7	1mM SA	T_{13}	25ppm Ethephon
T_2	300 ppm Potassium phosphonate	T_8	5mM SA	T_{14}	50ppm Ethephon
T_3	500 ppm Potassium phosphonate	T_9	10mM SA	T_{15}	100ppm Ethephon
T_4	10ppm ASM	T_{10}	25mM K_2HPO_4	T_{16}	1mM $FeCl_3$
T_5	25ppm ASM	T_{11}	50mM K_2HPO_4	T_{17}	5mM $FeCl_3$
T_6	50ppm ASM	T_{12}	100mM K_2HPO_4	T_{18}	10mM $FeCl_3$

Fig. 2. Effect of chemical activators on bacterial blight of anthurium

Application of 300ppm potassium phosphonate , 10ppm ASM and 10mM $FeCl_3$ recorded 43.65, 51.61 and 64.51 per cent bacterial blight infection which were also significantly superior over the check.

The effectiveness of 10mM SA, 100mM K_2HPO_4, 100ppm potassium phosphonate, 1mM and 5mM $FeCl_3$ against bacterial blight were statistically on par and recorded 77.28 to 73.45 per cent disease intensity. This was followed by 50mM K_2HPO_4, 100ppm ethephon and 10mM SA which were also on par and recorded slightly higher percentage disease intensity. The treatments 25mM and 50mM K_2HPO_4 and 100ppm ethephon were also on par with more than 80 per cent disease intensity but significantly better than the check.

Treatments such as 1mM SA, 5mM SA, 25mM K_2HPO_4, 25ppm and 50ppm ethephon had no effect on bacterial blight disease management in anthurium.

Out of the six chemical activators and their three doses, best two were selected for the next *in vivo* experiment. The best treatments were found to be 500 ppm potassium phosphonate, 25 ppm and 50 ppm ASM. Since the two doses of ASM were on par and showed more or less the same result, the lowest dose of 25ppm was included in the next *in vivo* study.

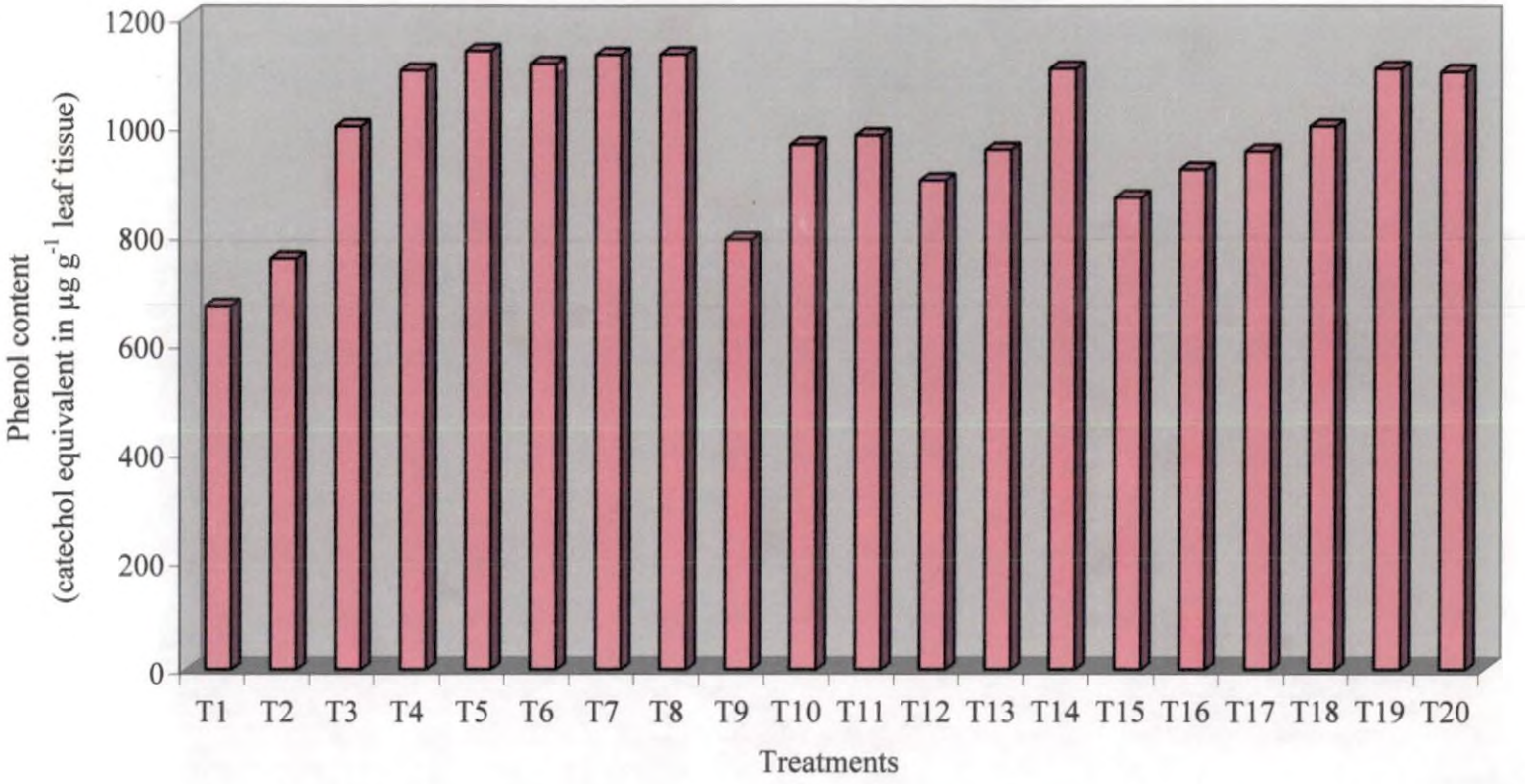
4.5.2.1 Biochemical Studies

4.5.2.1.1 Total Phenol

From the results presented in the table 8 and Fig. 3, it is evident that, the total phenol content is highest in the plants treated with 500ppm akomin *i.e.*, 1138.63 µg g^{-1} leaf tissue. This was followed by the phenol content in plants applied with 50ppm ASM and 25ppm ASM, the activity being 1132.00 and 1131.61µg g^{-1} leaf tissue respectively. The other treatments which recorded higher total phenol content (*i.e.*, above 1000 µg g^{-1} leaf tissue) were 10mM Fe Cl_3, 5mM Fe Cl_3, 100mM K_2HPO_4, 1mM $FeCl_3$

Table 8 Total Phenol content of the plants treated with chemical activators

Sl No.	Treatments	Phenol content (catechol equivalent in µg g^{-1} leaf tissue)
1	Uninoculated control	670.12
2	Inoculated control	756.66
3	Akomin 100ppm	1000.00
4	Akomin 300ppm	1102.24
5	Akomin 500ppm	1138.63
6	ASM 10ppm	1115.31
7	ASM 25ppm	1131.61
8	ASM 50ppm	1132.00
9	SA 1mM	792.00
10	SA 5mM	966.33
11	SA 10mM	983.33
12	K_2HPO_4 25mM	901.21
13	K_2HPO_4 50mM	957.00
14	K_2HPO_4 100mM	1106.00
15	Ethephon 25ppm	869.00
16	Ethephon 50ppm	921.22
17	Ethephon 100ppm	953.66
18	$FeCl_3$ 1mM	1000.00
19	$FeCl_3$ 5mM	1105.35
20	$FeCl_3$ 10mM	1100.00



T_1	Uninoculated control	T_6	ASM 10ppm	T_{11}	SA 10mM	T_{16}	Ethephon 50ppm
T_2	Inoculated control	T_7	ASM 25ppm	T_{12}	K_2HPO_4 25mM	T_{17}	Ethephon 100ppm
T_3	Akomin 100ppm	T_8	ASM 50ppm	T_{13}	K_2HPO_4 50mM	T_{18}	$FeCl_3$ 1mM
T_4	Akomin 300ppm	T_9	SA 1mM	T_{14}	K_2HPO_4 100mM	T_{19}	$FeCl_3$ 5mM
T_5	Akomin 500ppm	T_{10}	SA 5mM	T_{15}	Ethephon 25ppm	T_{20}	$FeCl_3$ 10mM

Fig. 3. Total Phenol content of the plants treated with chemical activators

and 100 ppm potassium phosphonate. Treatments such as 10mM SA, 25mM and 50mM K_2HPO_4, 50ppm ethephon, 5mM SA, 25ppm ethephon and 1mM SA had low total phenol content. The total phenol content of the inoculated control was lesser, 756.66 µg g^{-1} leaf tissue. The lowest phenol content was for uninoculated control *i.e.*, 670.12 µg g^{-1} leaf tissue.

4.5.2.1.2 Phenyl Alanine Ammonia Lyase (PAL)

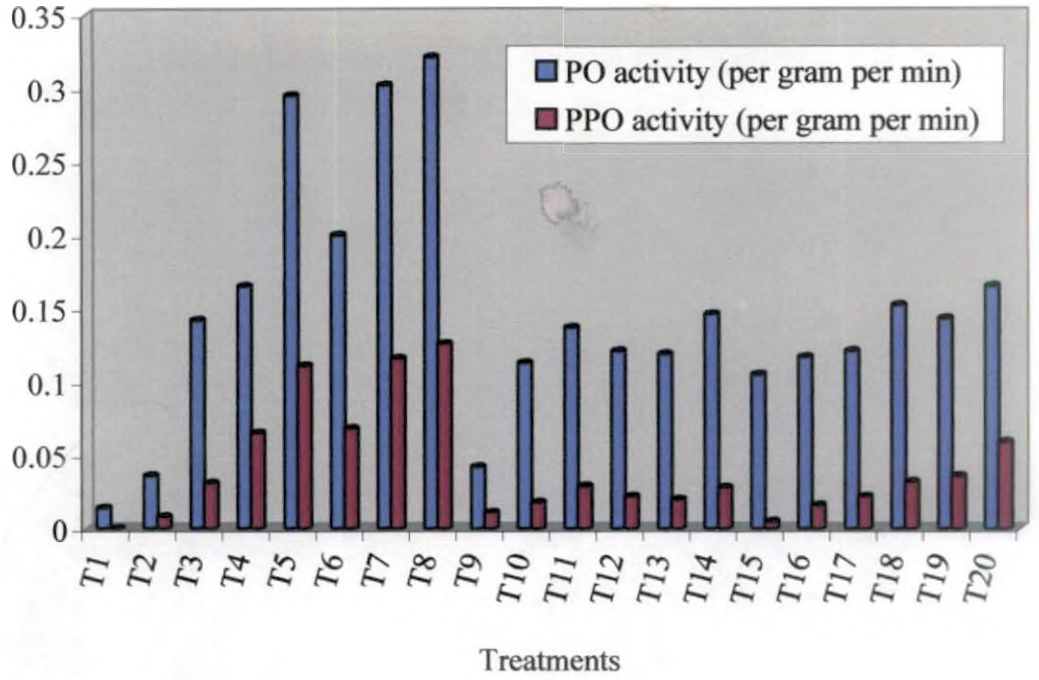
According to the data in the table 9 and Fig. 4, 25ppm and 50ppm ASM recorded the highest PAL activity, activity being 28.75 and 29.25 µg g^{-1} leaf tissue. It was followed by 500ppm potassium phosphonate (25.27µg g^{-1} leaf tissue). The other treatments showing high PAL activity were 10 ppm ASM, 300ppm potassium phosphonate, 10mM, 5 mM and 1mM $FeCl_3$, 100ppm potassium phosphonate, 10mM SA and 100mM K_2HPO_4. The treatments which recorded low PAL content included 25mM K_2HPO_4, 100ppm ethephon, 50mM K_2HPO_4; 1mM SA, 50ppm ethephon, 5mM SA and 25ppm ethephon. Inoculated control and uninoculated control had the least PAL activity *i.e.*, 8.15 and 7.95µg g^{-1} leaf tissue respectively.

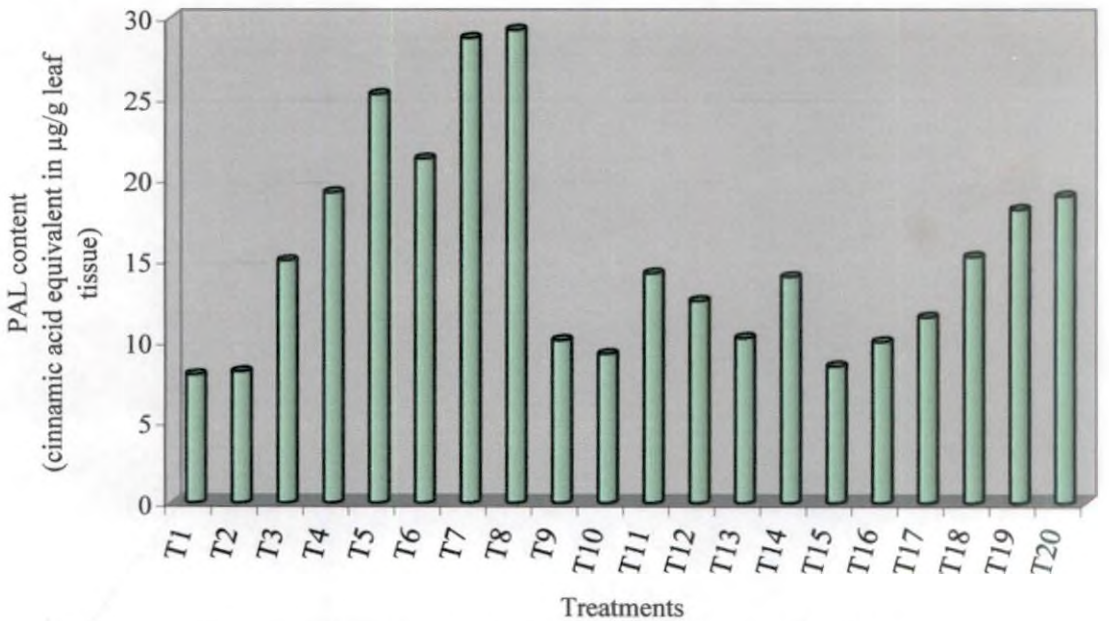
4.5.2.1.3 Peroxide (PO)

The data is presented in the table 9 and Fig. 4. The activity of peroxidase enzyme was higher in treated plants and in all the treatments except $FeCl_3$ the enzyme activity increased as the concentration of the chemicals increased. The peroxidase activity was highest in plants treated with 50ppm ASM *i.e.*, 0.321 g^{-1} min^{-1}. This was followed by 25ppm ASM and 500ppm potassium phosphonate, the activity being 0.302 g^{-1} min^{-1} and 0.295 g^{-1} min^{-1} respectively. The other treatments which recorded the higher PO activity were 10mM and 1mM $FeCl_3$, 100mM K_2HPO_4, 5mM $FeCl_3$, 100ppm potassium phosphonate and 10mM SA. Treatments such as 25mM and 50mM K_2HPO_4, 50ppm ethephon, 5mM SA, 25ppm ethephon and 1mM SA had low PO activity. Inoculated control showed still low PO

Table 9 Activity of defense related enzymes (PO, PPO and PAL) in the plants treated with chemical activators

Sl No.	Treatments	PO activity (per gram per min)	PPO activity (per gram per min)	PAL content (cinnamic acid equivalent in µg g^{-1} leaf tissue)
1	Uninoculated control	0.014	0.000	7.95
2	Inoculated control	0.036	0.008	8.15
3	Potassium phosphonate 100ppm	0.142	0.031	15.00
4	Potassium phosphonate 300ppm	0.165	0.065	19.20
5	Potassium phosphonate 500ppm	0.295	0.111	25.27
6	ASM 10ppm	0.200	0.068	21.27
7	ASM 25ppm	0.302	0.116	28.75
8	ASM 50ppm	0.321	0.126	29.25
9	SA 1mM	0.042	0.011	10.10
10	SA 5mM	0.113	0.018	9.25
11	SA 10mM	0.137	0.029	14.22
12	K_2HPO_4 25ppm	0.121	0.022	12.52
13	K_2HPO_4 50ppm	0.119	0.020	10.24
14	K_2HPO_4 100ppm	0.146	0.028	14.00
15	Ethephon 25ppm	0.105	0.005	8.50
16	Ethephon 50ppm	0.117	0.016	10.00
17	Ethephon 100ppm	0.121	0.022	11.52
18	$FeCl_3$ 1mM	0.152	0.032	15.25
19	$FeCl_3$ 5mM	0.143	0.036	18.17
20	$FeCl_3$ 10mM	0.165	0.059	19.00





T_1	Uninoculated control	T_{11}	SA 10mM
T_2	Inoculated control	T_{12}	K_2HPO_4 25mM
T_3	Akomin 100ppm	T_{13}	K_2HPO_4 50mM
T_4	Akomin 300ppm	T_{14}	K_2HPO_4 100mM
T_5	Akomin 500ppm	T_{15}	Ethephon 25ppm
T_6	ASM 10ppm	T_{16}	Ethephon 50ppm
T_7	ASM 25ppm	T_{17}	Ethephon 100ppm
T_8	ASM 50ppm	T_{18}	$FeCl_3$ 1mM
T_9	SA 1mM	T_{19}	$FeCl_3$ 5mM
T_{10}	SA 5mM	T_{20}	$FeCl_3$ 10mM

Fig. 4. Activity of defense related enzymes (PO, PPO and PAL) in the plants treated with chemical activators

activity, 0.036 g^{-1} min^{-1}. The lowest PO activity was expressed by uninoculated control *i.e.*, a mere 0.014 g^{-1} min^{-1}.

4.5.2.1.4 Polyphenol Oxidase (PPO)

As in the case of peroxidase, the PPO activity was triggered by artificial inoculation of the pathogen as well as application of different treatments on anthurium plants. According to the data in the table 9 and Fig. 4, 50ppm ASM recorded the highest PPO activity, activity being 0.126 g^{-1} min^{-1}. It was followed by 25ppm ASM and 500ppm potassium phosphonate (0.116 and 0.111 g^{-1} min^{-1} respectively). The other treatments showing high PPO activity were 10ppm ASM, 300 ppm potassium phosphonate, 10mM, 5mM and 1mM $FeCl_3$, 100ppm potassium phosphonate, 10mM SA and 100mM K_2HPO_4 . The treatments which recorded low PPO activity included 25mM K_2HPO_4, 100ppm ethephon, 50mM K_2HPO_4, 5mM SA, 50ppm ethephon, and 1mM SA. Inoculated control and uninoculated control showed the lowest PPO activity *i.e.*, 0.008 g^{-1} min^{-1} where as in uninoculated control there was no PPO activity.

4.6 MANAGEMENT OF BACTERIAL BLIGHT OF ANTHURIUM

Ten month old tissue culture anthurium plants of variety Cancan were used for the experiment. The treatments were given as root dip two weeks prior to pathogen inoculation. One week later, these treatments were given as foliar spray. The results of the trial are presented in table 10 and Fig. 5.

The plants which were treated with *Bacillus* sp. and 0.15 per cent of turmeric powder + sodium bicarbonate (10:1) developed no infection whereas the control plants recorded 69.57 per cent disease intensity (Plates 6a and 6b).

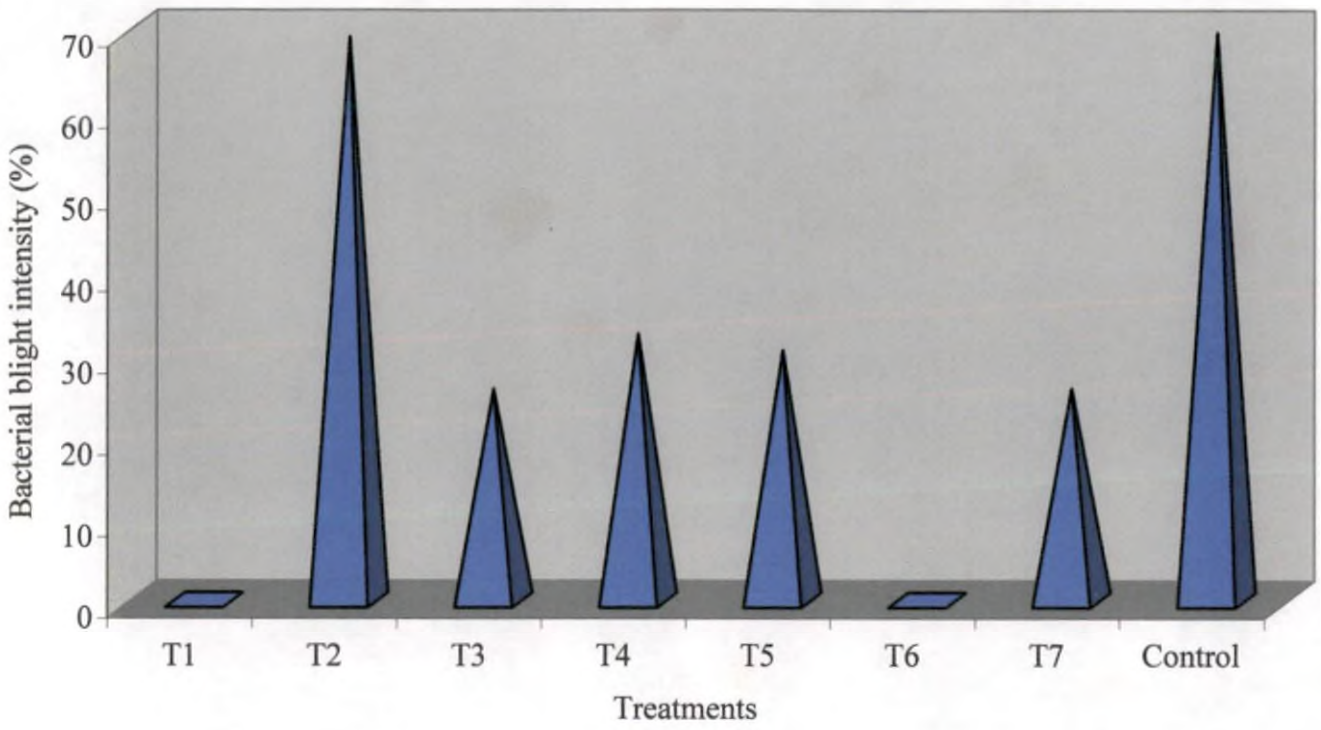
Table 10 Effect of root dip and foliar spray of chemical activators and antagonists on bacterial blight of anthurium

Sl No.	Treatments	Bacterial blight intensity (%)	Per cent reduction over control
1	*Bacillus* sp.	0 (0)	100
2	*Talaromyces* sp.	69.08 (56.19)	0.704
3	Potassium phosphonate 500ppm	25.98 (30.63)	62.66
4	ASM 25ppm	32.72 (34.88)	52.97
5	Crude extract of neem cake	30.70 (33.63)	55.87
6	0.15 per cent of turmeric powder + sodium bicarbonate (10:1)	0 (0)	100
7	Streptocycline 100ppm	26.07 (30.69)	62.53
8	Control	69.57 (56.50)	0

*Average of three replications

Figures in parenthesis are transformed values (angular transformation)

CD=2.2



T_1 *Bacillus* sp.
T_2 *Talaromyces* sp.
T_3 Potassium phosphonate 500ppm
T_4 ASM 25ppm
T_5 Crude extract of neem cake
T_6 0.15% of turmeric powder + sodium bicarbonate (10:1)
T_7 Streptocycline 100ppm

Fig. 5. Effect of root dip and foliar spray of chemical activators and antagonists on bacterial blight of anthurium

Plate 6a. Effect of treatment of *Bacillus* sp. (B_{16}) on bacterial blight of anthurium

Plate 6b. Effect of treatment of 0.15 per cent of turmeric powder + sodium bicarbonate (10 : 1) on bacterial blight of anthurium

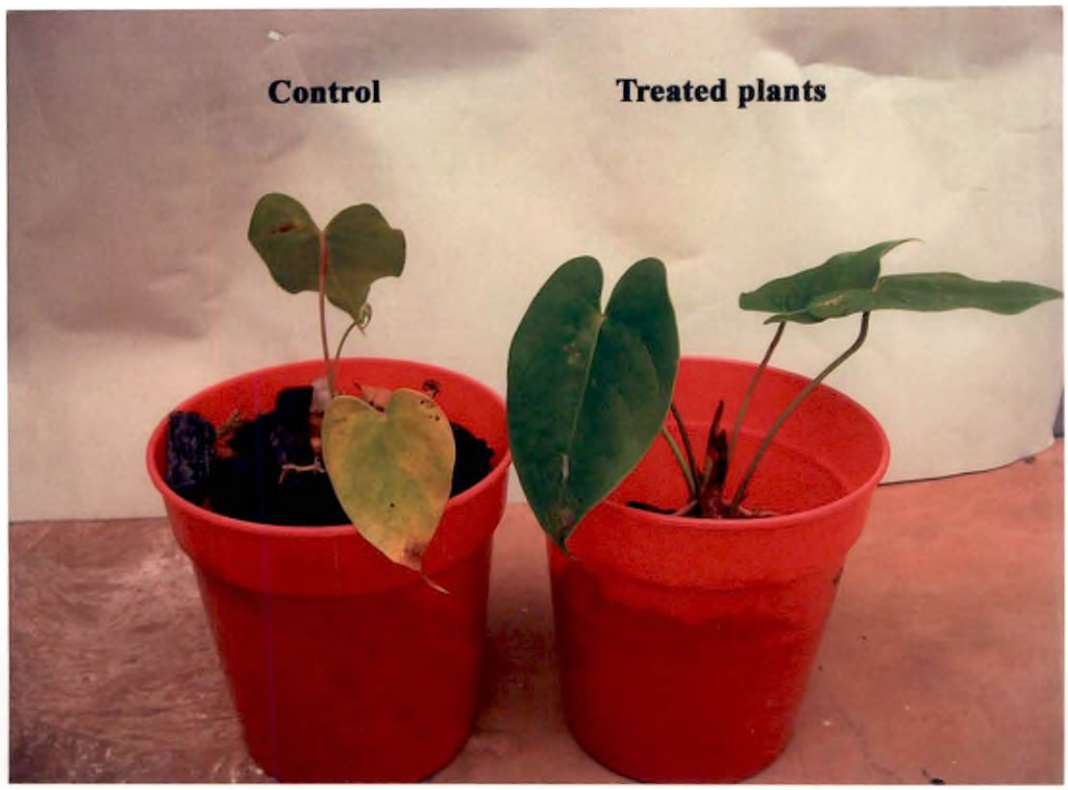


Plate 6a.

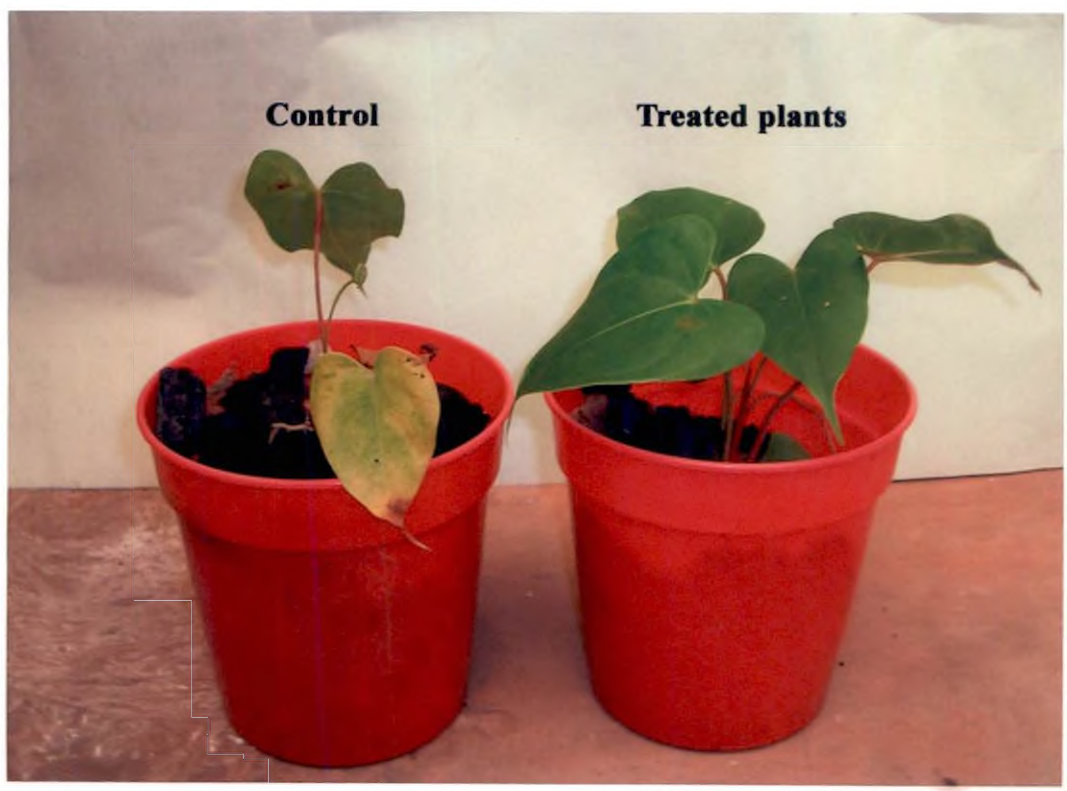


Plate 6b.

Streptocycline (100ppm) and 500ppm potassium phosphonate treated plants developed 26.07 and 25.98 per cent disease intensity respectively which were statistically on par with each other and significantly superior to control plants.

Treatments 25ppm ASM and crude extract of neemcake were also effective for the management of bacterial blight disease as the treatments recorded 32.72 and 30.70 per cent disease intensity respectively which were also on par with each other and significantly superior than the control plants.

The antagonistic fungus *Talromyces* sp. had no effect on bacterial blight disease under field conditions.

Thus root dip and foliar spray of the bacterial antagonist *Bacillus* sp. and 0.15 per cent turmeric powder + sodium bicarbonate (10:1) were found to be far superior for the management of bacterial blight of anthurium. Streptocyclin (100ppm) and potassium phosphonate (500ppm) ranked second followed by ASM (25ppm) and crude extract of neemcake. Bacillus sp. used in this experiment is a native isolate, the antagonistic property of which was established against a virulent isolate of *Xanthomonas axonopodis* pv. *dieffenbachiae*, the pathogen of bacterial blight.

Since there is no effective control measures at present against this disease except antibiotics which on repeated application was reported to develop resistance against the pathogen, the locally isolated bacterial antagonist, *Bacillus* sp. and the ecofriendly material turmeric powder which is widely available in our state could be effectively used and recommended for bacterial blight disease management.

Discussion

5. DISCUSSION

The efficacy of an alternate management strategy using microbial antagonists and resistance inducers for the management of bacterial blight of anthurium was investigated in the present study. *Xanthomonas axonopodis* pv. *dieffenbachiae* is the incitant of the bacterial blight of anthurium. This disease was first reported from Brazil in 1960. In India, the occurrence of the same was reported in imported anthurium plants by Sathyanarayana *et al.* (1998). The local infection is incited as irregular water soaked lesions on leaf margins and lamina which later on increase in size and turn dark brown surrounded by a yellow halo. The systemic infection is initially manifested in the collar region which eventually leads to rotting of the entire plant. Antibiotics like streptomycin have been found to be effective against *X. axonopodis* pv. *dieffenbachiae* (Sato, 1983; Barret and Cassels,1994). But Knauss (1972) reported that continuous applications have resulted in the emergence of resistance to streptomycin. A search for other potential methods to manage this disease has led to the present investigation.

Nowadays much emphasis is being given for eco-friendly disease management strategy using microbial antagonists. This strategy has been found to be quite successful in greenhouse crops as they are grown in a closely monitored environment which favours the success of this method. Therefore biological control seems to be a promising alternative for a green house crop like anthurium.

In the present study the pathogen inciting bacterial blight of anthurium, *X. axonopodis* pv. *dieffenbachiae* was isolated and Koch's postulates were proved. A virulent isolate of the pathogen was used for further studies.

As part of this investigation, microflora from both rhizosphere and phyllosphere of healthy anthurium plants were screened for their efficacy in inhibiting the growth of bacterial blight pathogen. Among them a bacterial antagonist identified as a species of *Bacillus* (B_{16}) and a fungal antagonist identified as a species of *Talaromyces* (F_8) recorded maximum growth inhibition of *X. axonopodis* pv. *dieffenbachiae*. The antagonistic efficiency of Bacillus sp against a wide spectrum of phytopathogens has already been established (Broadbent *et al.*, 1971; Weller, 1988; Singh and Rana, 2000; Pradeepkumar and Sood, 2001). The antagonistic effect of Bacillus sp. against pathogens belonging to the genus Xanthomonas has also been investigated earlier (Arya and Parashar, 1998; Hong *et al.*, 1999; Wuff *et al.*, 2002; Hong *et al.*, 2003; Massomo *et al.*, 2004).

In the present study, next to B_{16} *(Bacillus* sp), the fungi *Talaromyces* exhibited maximum inhibition to *X. axonopodis* pv. *dieffenbachiae*. Antagonistic efficiency of fungal antagonists against fungal pathogens has already been reported (Weindling, 1932; Gokulapalan and Nair, 1984; Mukherjee and Sen, 1992; Singh and Singh, 2000). But reports of fungal antagonists against bacterial pathogen are very meagre. There is not much evidence regarding any earlier work connecting *Talaromyces* and *X. axonopodis* pv. *dieffenbachiae*. In the present study much of the inhibition of the pathogen was due to the production of some compounds by *Talaromyces* as a clear zone was produced around the fungal disc. Stosz et al (1998) have reported that the enzyme glucose oxidase was involved in the biocontrol of *Verticillium* by *Talaromyces flavus*.

The next part of the management strategy adopted in this investigation was using chemical activators. Chemical activators are a group of defined synthetic chemicals that are activators of disease resistance in plants with pre-challenge inoculation. These are non-fungitoxic and are active at low concentration. Since they trigger host

defense activity, they are less likely to encounter a problem of development of resistance by the pathogen. These compounds are potentially valuable since the side effects of microbial balance of the environment are less severe. Many reports exist in literature about chemicals with resistance inducing activity (Metraux *et al.*, 1991; Kunz *et al.*, 1997).

In the present investigation, six different chemicals *viz.*, potassium phosphonate, acibenzolar-s-methyl, salicylic acid, di-potassium hydrogen phosphate, ethephon and ferric chloride were chosen. They were initially tested for any direct action on the pathogen, *X. axonopodis* pv. *dieffenbachiae*. None of them inhibited the pathogen *in vitro*. This is in conformity with the criterion laid down by Sticher et *al* (1997) that neither the agent nor its metabolite should have direct antifungal activity or antibacterial activity *in vitro*.

Following the *in vitro* study of the chemical activators, the in vivo study was also conducted. All the six chemical activators were screened against bacterial blight on six month old anthurium plants of variety Cancan. Minimum bacterial blight disease intensity of 12.93 percent was observed in plants treated with potassium phosphonate @ 500 ppm (Table 7; Fig.2). This was followed by 25 ppm and 50 ppm ASM. All the other treatments showed higher bacterial blight intensity. So from the above study two treatments *viz.*, 500 ppm potassium phosphonate and 25 ppm ASM were adjudged as the best. The ability of the ASM as a broad spectrum chemical activator has been established earlier (Friedrich *et al.*, 1996; Gorlach *et al.*, 1996; Kunz *et al.*, 1997). There are also reports regarding the control of *Xanthomonas* by ASM. Romero *et al.* (2001) reported the effectiveness of ASM in resisting infection by *X. axonopodis* pv. *vesicatoria* in bell pepper plants. Bounaurio *et al.* (2002) also reported the effectiveness of ASM in protecting pepper against *X. campestris pv. vesicatoria.* Similarly there are reports regarding the significance of

potassium phosphonate in combating diseases affecting black pepper and anthurium (Veena and Sarma, 2000; KAU, 2002).

To further ascertain the biochemical reasons in the trend followed by chemical activators in managing bacterial blight of anthurium, biochemical tests were conducted in this investigation. The phenol content of all the treatments was probed. The Fig (3) reveals that the highest phenol content was for 500 ppm potassium phosphonate treated plants. This was followed by ASM at 25 and 50 ppm. This result is in conformity with the previous *in vivo* experiment. All the selected treatments had high phenol content with respect to other treatments. So one of the reasons for the reduction in percent intensity of bacterial blight of anthurium is the increased phenol content. Phenol compounds are known to be associated with the resistance of red onion varieties against *Colletotrichum circinans* (Walker and Stahmann, 1995). So 500 ppm potassium phosphonate and 25 ppm ASM incited phenolic compounds and they in turn conferred resistance to plants.

Apart from total phenol content, the activity of defense related enzymes were also investigated in this study. The defense related enzymes studied were peroxidase, polyphenol oxidase and phenyl ammonia lyase. The enzyme, phenyl ammonia lyase (PAL) is involved in phytoalexin or phenolic compound biosynthesis (Beaudoin-Eagan and Thorpe, 1985).The oxidative enzymes on the other hand, such as peroxidase (PO) and polyphenol oxidase (PPO) catalyse the formation of defense barriers for reinforcing the cell structure (Avdiushko *et al.*, 1993). In the present study, the highest activity of all these enzymes were recorded for the treatments, 50 ppm ASM, 25 ppm ASM and 500 ppm potassium phosphonate. From the graph (Fig. 4) it is clear that all these three treatments showed a peak. The highest peak was obtained with 500 ppm ASM for all the enzymes *viz.*, PO, PPO and PAL. The treatments which scored a higher value next to 500 ppm potassium phosphonate were 25ppm

and 50 ppm ASM. For the next part of the study, the best two chemical activators *viz.*, 500 ppm potassium phosphonate and 25 ppm ASM were chosen as they showed minimum bacterial blight intensity. From the biochemical tests also it is clear that these two treatments had commendable biochemical reasons to justify their action *in vivo*. Eventhough 50 ppm ASM also showed minimum bacterial blight intensity, statistically it was on par with 25 ppm ASM. So the lower economical dose was selected.

The final part of the investigation in the management of bacterial blight of anthurium was an in *vivo* study. The treatments were, two efficient antagonists identified from the previous *in vitro* studies *ie.*, *Bacillus* sp. and *Talaromyces* sp., two best chemical activators viz., 500 ppm potassium phosphonate and 25 ppm ASM, crude extract of neem cake, 0.15 percent of turmeric powder + sodium bicarbonate (10:1), streptomycin @ 100 ppm and control. All the treatments were given as root dip and foliar spray prior to challenge inoculation.

Hundred percent disease reduction was obtained (Table 10; Fig. 5) with the treatment of Bacillus sp. and 0.15 percent turmeric powder + sodium bicarbonate (10:1). This was followed by 62.53 and 62.66 percent disease reduction over control in treatments, 100 ppm streptocycline and 500 ppm akomin. The treatments 25 ppm ASM and crude extract of neem cake were able to bring a disease reduction of 52.97 and 55.87 percent over control. The antagonistic fungus, *Talaromyces* sp. could reduce the disease by only 0.704%.

Similar reports on the management of *Xanthomonas* by *Bacillus* sp. as well as 0.15 percent turmeric powder + sodium bicarbonate (10:1) were obtained earlier. There were reports suggesting the efficacy of *Bacillus* sp. in managing pathogen belonging to the genus, *Xanthomonas*. Massomo *et al.* (2004) observed that strains of *Bacillus cereus, B. lentimorbus and B. pumilis* greatly reduced both incidence and severity of black rot of

cabbage caused by *X. campestris pv. campestris.* Dhanya (2000) observed that the treatment. 0.15 percent turmeric powder + sodium bicarbonate (10:1) was the best in managing bacterial blight of anthurium caused by *X. axonopodis* pv. *dieffenbachiae.*

The two effective management strategies identified in the present investigation are ecofriendly in nature. But the efficiency of antagonists must further be evaluated under field conditions. Further studies such as their effect on microflora and fauna, efficient delivery systems, safety towards other living beings etc. has to be carried out before commercializing it. The other strategy using turmeric powder and sodium bicarbonate is quite relevant as this technology as well as the components are widely available in our state and hence can be practiced by the anthurium growers.

Summary

6. SUMMARY

Anthurium (*Anthurium andreanum* Linden) is one of the most important cut flower crops of Kerala. It is infected by several diseases which seriously affect their productivity. Bacterial blight is one of the most serious disease of anthurium which affects the leaves, spathes and spikes and when it assumes systemic nature it can even kill the plants. The present investigation was undertaken to evolve a suitable management strategy for bacterial blight using microbial antagonists and chemical activators.

Infected plants were collected from different locations of Thiruvananthapuram district and the pathogen, *Xanthomonas axonopodis* pv *dieffenbachiae* was isolated from the affected parts. Out of different isolates of the pathogen obtained, Xad 6 was the most virulent one. It was used for all the further studies.

Microbial antagonists residing in the rhizosphere and phyllosphere were assessed for their potential in controlling *X. axonopodis* pv *dieffenbachiae*. Rhizosphere and phyllosphere microflora of healthy anthurium plants were isolated and were screened for their efficacy in inhibiting *X. axonopodis* pv *dieffenbachiae in vitro*. Among them two isolates- B_{16} and F_8 were observed to be superior in inhibiting the pathogen. B_{16} was identified as a species of *Bacillus* and F_8, a species of *Talaromyces*.

B_{16} (*Bacillus* sp.) was grown on seven different solid media. It was found that the growth was superior in nutrient agar, moderate in potato sucrose agar, potato dextrose agar and King's B agar and scanty in yeast extract glucose chalk agar, glucose agar and glucose yeast extract agar. For B16 (*Bacillus* sp.) the growth dynamics was also studied and their optical densities were recorded at 485 nm in peptone water. The isolate

was also tested for pathogenicity in anthurium plants. The tests proved negative for pathogenicity. The other antagonist, *Talaromyces* sp. (F_8) had a white fluffy growth and on initiation of sporulation the fluffiness was replaced by yellow and resin coloured spores.

Chemical activators *viz.*, potassium phosphonate, acibenzolar-s-methyl, salicylic acid, di-potassium hydrogen phosphate, ethephon and ferric chloride were tested against *X. axonopodis* pv *dieffenbachiae* at three different doses under *in vitro* conditions to check for any direct action of these chemicals on the pathogen. None of these chemicals at any of the doses exhibited any direct action on the pathogen as no inhibition zone was produced around the filter paper discs dipped in chemical activators on the pathogen seeded medium.

All the above mentioned chemical activators at their three doses were studied for their effect on the pathogen under *in vivo* conditions on six month old anthurium plants of variety Cancan. All of the treatments were given as a foliar spray one week prior to pathogen inoculation. From this study, two best treatments were selected for the final experiment as they recorded the least percentage disease intensity. The selected treatments were 500ppm potassium phosphonate and 25ppm acibenzolar-s-methyl.

A green house trial was conducted with ten month old tissue culture plants of variety Cancan with treatments given as root dip and foliar spray two weeks and one week prior to pathogen inoculation. Treatments included B_{16} (*Bacillus* sp), F_8 (*Talaromyces* sp.), 500ppm potassium phosphonate , 25ppm acibenzolar-s-methyl, crude extract of neem cake, 0.15% of turmeric powder + sodium bicarbonate (10:1) and 100ppm streptocycline. Treatments which recorded the least disease intensity were B_{16} (Bacillus sp) and 0.15% of turmeric powder +sodium bicarbonate (10:1). Both of them were equally effective in checking the disease. The chemical activators on other hand were next only in effectiveness to them.

This investigation thus not only put forward an alternative strategy to manage the disease but also confirms an earlier finding for effective management for bacterial blight of anthurium which could be readily recommended to anthurium growers.

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*Original not seen

Appendix

APPENDIX – I

BUFFERS FOR ENZYME ANALYSIS

A) 0.1 M Sodium phosphate Buffer (pH 6.4)

Stock solutions

A – 1.56 g of sodium dihydrogen phosphate in 100 ml.

B – 1.42 g of disodium orthohydrogen phosphate in 100 ml.

68.5 ml A is mixed with 31.5 ml B

B) 0.1 M Sodium Borate Buffer (pH 8.8)

Stock solutions

A – 0.2 M solution of boric acid (12.4 g in 1000 ml)

B – 0.05 M solution of borax (19.05 g in 1000 ml)

50 ml of A is mixed with 30 ml of B, diluted to a total of 200 ml.

MICROBIAL ANTAGONISTS AND RESISTANCE INDUCERS FOR THE MANAGEMENT OF BACTERIAL BLIGHT OF ANTHURIUM (*Anthurium andreanum* Linden)

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Abstract of the
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ABSTRACT

An investigation was undertaken for exploring the potential of microbial antagonists and chemical activators for the management of bacterial blight of anthurium. Different isolates of the pathogen, *Xanthomonas axonopodis* pv. *dieffenbachiae* were collected and Xad 6, the most virulent one was selected for further studies.

The microbial flora from the rhizosphere and phyllosphere of healthy anthurium plants from various locations were isolated and screened for their efficacy in inhibiting *X. axonopodis* pv.*dieffenbachiae* under *in vitro* conditions. Among the microbes obtained, isolates of *Bacillus* sp. (B_{16}) and *Talaromyces* sp. (F_8) were the most effective. Nutreint agar proved to be the best media for B_{16} and the antagonist was also non-pathogenic to anthurium. The potential of chemical activators in management of bacterial blight of anthurium was also investigated both under *in vitro* and *in vivo* conditions. The *in vitro* studies revealed that chemical activators had no direct action on the pathogen, conforming to one of the characteristics of an activator for inducing disease resistance in plants (Systemic acquired resistance, SAR). Among the chemical activators tested under *in vivo* conditions, 500ppm potassium phosphonate and 25ppm acibenzolar-s-methyl showed considerable effect in reducing the per cent disease intensity.

The best treatments from the above studies and some other treatments which exhibited good results against bacterial blight of anthurium from previous works were studied in a green house trial on ten month old tissue culture anthurium plants of Cancan variety. The treatments included B_{16}, F_8, 500 ppm potassium phosphonate, 25 ppm acibenzolar-s-methyl, crude extract of neem cake, 0.15 per cent of turmeric powder + sodium bicarbonate (10:1) and 100 ppm streptocycline.

The result indicated that the treatments, B16 (Bacillus sp) and 0.15 per cent of turmeric powder +sodium bicarbonate (10:1) were effective as they showed least bacterial blight intensity.

So it can be concluded that, a prophylactic root dip and foliar spray of *Bacillus* sp. (B_{16}) or turmeric powder + sodium bicarbonate (10:1) @ 0.15 per cent were equally effective in managing bacterial blight of anthurium. The role of chemical activators on the other hand in combating the disease was next only to microbial antagonists and ecofriendly material.