# POTENTIAL OF ANTAGONISTIC ENDOPHYTES AGAINST BACTERIAL BLIGHT OF ANTHURIUM

**By**

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**THESIS**

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***for the degree of***

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## DECLARATION

I, **Sanju Balan(2006-11-118)**,hereby declare that this thesis entitled **"Potential of antagonistic endophytes against bacterial blight of anthurium"** is a *bonafide* record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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## CERTIFICATE

Certified that this thesis entitled **"Potential of antagonistic endophytes against bacterial blight of anthurium"** is a *bonafide* record of research work done independently by **Mrs. Sanju Balan(2006-11-118)** under my guidance and supervision and that it has not formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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# *Introduction*

# 1. INTRODUCTION

Anthurium is one of the important commercial cut flower crops of Kerala. It is a native of South West Columbia and is widely seen in many parts of world. Anthurium is now being cultivated in many parts of India including Kerala, as the flower has got good market. Further, the climatic conditions are ideal for growing the plants in the state. As in the case of other crops, anthurium is also prone to many fungal, bacterial and viral diseases. Among these, bacterial blight incited by *Xanthomonas axonopodis* pv *dieffenbachiae* is a major constraint affecting the cultivation of anthurium. It was reported for the first time from Kauai islands in Hawaii. In Kerala, the disease was first reported by Dhanya (2000).

Current management practices against the disease mainly involve use of antibiotics. However, continuous use of antibiotics may result in the emergence of resistant strains of the pathogen which may pose many problems in future. As an alternative and sustainable strategy, the use of bioagents, especially that of endophytic microbes can be exploited. Nowadays management using biocontrol agents is gaining momentum as they are eco friendly and cost effective. In addition, the endophytes have an additional advantage of inhabiting internal environment of plant which ensures protection and nutrient supply. The chance of development of resistance is also negligible. Apart from suppressing the pathogens by production of inhibitory metabolites, they are capable of promoting plant growth and induce systemic resistance. The possibility of using plants own defense mechanisms, induced by endophytes, in the management of pest and diseases is a matter of current interest. Further, among the biocontrol agents, endophytes are found to be more reliable and economically sustainable. Recognizing the potentiality of this field and also the fact that bacterial blight is the sole major constraint in the cultivation of anthurium, the present study was undertaken with the following objectives.

1. Isolation and characterization of the pathogen
2. Isolation and enumeration of the endophytic micro organisms of anthurium
3. Testing *in vitro* antagonistic effect of endophytes against the pathogen
4. Testing compatibility of selected antagonists with common plant protection chemicals, nutrients etc.
5. Studying mode of action of endophytes
6. Characterization of endophytes
7. *In vivo* effect of selected endophytes against bacterial blight pathogen

# *Review of literature*

## 2. REVIEW OF LITERATURE

Anthurium, an important cut flower crop, belongs to the family Araceae. One of the most serious threats to anthurium cultivation is the incidence of bacterial blight. The disease assumes serious proportions during warm humid condition (Nishijima and Fujiyama, 1985). Mc culloch and Pirone (1939) first described the disease on Dieffenbachiae and named the pathogen as *Bacterium dieffenbachiae.* According to Hayward (1972), bacterial blight of anthurium was observed in early 1970s at Kauai Islands, Hawaii. Since then the disease was reported from California (Cooksey 1985), Venezuela (Guevara and Debrot, 1984), Florida (Hoogasian, 1990), Philippines (Natural, 1990), Jamaica (Young, 1990), Italy (Zoina *et al*., 2000), Reunion Island (Soustrade *et al.*, 2000), Turkey (Aysan and Sahin, 2003), New Caledonia (Jouen *et al*., 2007) and Poland (Pulawska *et al*., 2008).

In India, occurrence of this disease was reported in imported anthurium plants by Satyanarayana *et al*. (1998). The pathogen was then named as *Xanthomonas* species by Das *et al*. (1996). Later, it was reported from Kerala by Dhanya (2000) and identified as *Xanthomonas axonopodis* pv. *dieffenbachiae*.

### 2.1 Symptamatology

Hayward (1972) described the symptoms of the disease in detail. According to him, the most consequent symptom of the disease was angular pale brown necrotic spots with chlorotic halo on older leaves. On younger leaves lesions were dark brown, variable in size and vein delimited often extend along midrib. Similar symptoms were observed on spathe also. In contradiction to this, Lipp *et* a*l* .1992 noted that oldest and youngest leaves were least affected. Later in 1984, Guevara and Debrot reported the symptom of bacterial blight. They described the initial symptom as necrotic water soaked spots with chlorotic halo mainly towards the margin of leaves and spathe. Later the infection spreads through the petiole resulting in death of the plants. Both systemic and foliar

symptoms were explained by Natural *et al*. (1990). Foliar symptom appeared as minute water soaked lesions on undersurface of leaf which often became necrotic with bright yellow border. Systemic infection was noticed as yellowing of plants. The base of infected petioles when cut length wise, showed distinct brown spots. As a result of systemic infection entire plant got killed. Such types of symptoms were also reported by many other workers (Chase 1988, Kuehnle *et al*.,1995., Das *et al*., 1996 and Dhanya, 2000).

**2.2 Pathogen**

Many workers had studied the characters of the causal agent of bacterial blight of anthurium, *Xanthomonas axonopodis* pv. *dieffenbachiae* (Mc culloch and Pirone, 1939; Dye, 1980 and Vauterin *et al*., 1995). According to Hayward (1972), the pathogen was motile, gram negative and produced yellow growth in 2 per cent peptone sucrose agar. The bacterium was strictly aerobic and produced acid oxidatively from arabinose, cellobiose, fructose, galactose, glucose, glycerol, lactose, maltose, mannose, mannitol, raffinose, sucrose and tetrahalose whereas no acid was produced from dulcitol, inositol, salicin and sorbitol. It hydrolysed starch, lipid and gelatin and grew in media containing 2 to 3 per cent sodium chloride but not at higher concentrations. Guevara and Debrot (1984) noted that, the pathogen was rod rod shaped 0.4 – 0.6 X 1.0 X 2.4µm in size and with a polar flagellum. According to Pohronezny *et al*. (1985), colonies of the pathogen were yellow and mucoid on Nutrient Agar and Yeast Extract Dextrose Calcium Carbonate Agar. The isolate was catalase positive, produced hydrogen sulphide, liquefied gelatin, hydrolyzed aesculin and grew well at $35^0$C. Marked proteolysis occurred in litmus milk. They were oxidase negative, did not produced indole and no urease activity and reduction of nitrite was observed. Asparagine was not utilized as a sole source of carbon and nitrogen. Most isolates produced acid from glucose, arabinose, cellobiose, fructose, galactose, maltose, mannose, raffinose, sucrose and tetrahalose, but failed to produce acid from glycerol, mannitol, dulcitol, inositol, insulin, rhamnose, salicin and sorbitol.

Alvarez *et al*. (1990) reported the existence of two distinct biological types of *Xanthomonas axonopodis* pv. *dieffenbachiae,* one which grew fast and digest starch and the other slower growing which cannot digest starch. Lipp *et al*. (1992) observed that 62 per cent of the anthurium strain of *Xanthomonas axonopodis* pv. *dieffenbachiae* did not hydrolyse starch as compared to seven percent of strains obtained from other host. Dhanya (2000) reported that, bacterium was aerobic. The bacteria produced $H_2S$, $NH_3$, lipase and liquefied gelatin whereas it showed negative reaction for methyl red test, urease test, arginine dihydrolase and tyrosinase activity. None of the isolates tested utilized asparagine as sole source of carbon and nitrogen and none were tolerant to six percent sodium chloride. Isolates varied with regard to starch hydrolysis, growth characters and virulence.

**2.3 Management**

Many attempts were made by various workers to reduce the incidence and severity of bacterial blight of anthurium. Effectiveness of cultural measures like sanitation, disinfecting harvesting implements, reducing water splash, culling diseased plants and removing alternate host demonstrated were reported. (Nishijima and Fujiyama,1985; Lipp *et al*., 1992). Chase (1992) reported that lowering fertilizer rate for potted anthurium reduced blight incidence. Growing plants under plastic or glass house coupled with drip irrigation rather than over head sprinkler irrigation reduced the spread of bacteria through aerosols and water splash and significantly reduced the incidence of blight in anthurium (Kamemoto and Kuehule, 1989). Sakai (1990) opined that lowering the level of amino compounds in guttation fluid by use of nitrate fertilizers reduced the level of blight infection.

**2.3.1 Chemical control**

Many attempts were made by various workers to manage the disease using antibiotics. According to Nishijima (1989), exposure to Oxytetracycline at 32 ppm

for 16 days inhibited anthurium bacterial blight pathogen under *in vitro* conditions. Further he obtained good control of the disease in *in vivo* by drenching plants with Oxytetracycline at 1000 or 1500 ppm 3 or 4 times at weekly intervals. Hseu and Lin (1998) reported that, dithiocarbamate pesticides and Tetracycline inhibited the growth of *Xanthomonas axonopodis* pv *dieffenbachiae*. Dhanya (2000) who studied the etiology and management of bacterial blight of anthurium could get effective control of anthurium bacterial blight using captan 0.3 per cent and Streptocycline 100 ppm under *in vitro* condition. Valsalakumari and Rajeevan (2007) reported that, bacterial blight of anthurium was controlled under field condition by spraying the plants with Streptomycin sulphate or Oxytetracycline 200 ppm weekly for 6 weeks.

Turmeric sodium bicarbonate mixture was reported as potent biocontrol agent against rice bacterial blight and other fungal diseases.(Gangopadhyay,1998) Dhanya (2000) opined that, application of turmeric powder impregnated with sodium bicarbonate in the ratio of 10:1 gave the best result and was on par with Streptocycline. She also noticed ineffectiveness of cow dung extract against the pathogen both under field and lab conditions. Sabitha (2002) found that the *Allium sativum* extract was the best among tested botanicals against bacterial blight *in vitro*. Among oils and plant products, neem cake and neem oil were found effective against the pathogen. *In vivo* studies revealed the effectiveness of crude extracts of neem cake and *Tagetus erecta* against the bacterial blight pathogen.

Many studies were carried out on the sensitivity of antibiotics against pathogenic Xanthomonads. Padhya *et al*. (1963) studied the sensitivity of different species of *Xanthomonas* to various antibiotics and reported that all isolates were sensitive to tested antibiotics *viz.,* Streptomycin, Tetracycline, Oxytetracycline, Neomycin, Chloramphenicol and Kanamycin but not to Penicillin. Among seven antibiotics tested against *Xanthomonas oryzae*, only Chloromycetine and Streptocycline at 400 and 500ppm totally inhibited lesion development (Devadath, 1973)

Strider (1975) studied the chemical control of bacterial blight of begonia and reported that, Streptomycin sulphate at 600 ppm gave the best control, followed by Cupric hydroxide 86 W at 6 1b/1000 gal applied 1 day before inoculation. Thind and Mehra(1992) reported that application of Plantomycin,Streptocycline ,bleaching powder and zinc sulphate as preplanting nursery dip reduced the disease intensity of *Xanthomonas oryzae pv. Oryzae*.Gupta (1997) evaluated antibiotics and fungicides against *Xanthomonas cyamopsidis* causing bacterial blight of guar. Streptocycline at 100-250 ppm and Agrimycin 100 (Streptomycin + Oxytetracyclin) at 100-500 ppm gave the best results. Maximum inhibition of rice bacterial blight pathogen under *in vitro* condition was achieved with Streptocycline among the two antibiotics and eight fungicides tested (Mahto *et al*., 1988).

**2.3.2 Biological control**

Biological control is the reduction of inoculum density or disease producing ability of pathogen or parasite in its active or dormant state by one or more organisms accomplished naturally or through manipulation of environment, host or antagonists or by mass introduction of one or more antagonists (Baker and Cook, 1974).

Potential agents for biocontrol activity are rhizosphere competent fungi and bacteria which in addition to their antagonistic activity are capable of inducing growth response by either controlling minor pathogens or producing growth stimulating factors (Weller *et al*., 1988). Moreover, biocontrol agents, being ecofriendly, is more attractive proposition of crop protection especially where products are export oriented. In contrast to agrochemicals which get leached off during incessant rains, biocontrol agents get stabilized once efficient strain that fit into the concerned ecological niche are introduced into a given environment. Also, biocontrol agents fit well with organic farming, a proposition

which is giving popularity in recent times. Considerable progress has been made in the biological control of bacterial blight since it was first studied by Fernandez *et al*. (1989). Fukui *et al*. (1999) studied the management of bacterial blight of anthurium by bacterial community isolated from guttation fluid. They reported that inhibitory effect was related to the species of bacterial community rather than total number of bacteria in guttation fluid. Anjana (2005) who studied the management of bacterial blight of anthurium using microbial antagonists and resistance inducers found that foliar spray of *Bacillus* sp or Turmeric powder impregnated with sodium bicarbonate were equally effective in managing bacterial blight of anthurium.

Usefulness of biocontrol agents in the management of various bacterial diseases of crop plants have been demonstrated. Phylloplane bacteria and fungi were found effective in managing *X. campestris* pv. *cymopsidis* (Parashar *et al*.. 1992) and *Xanthomonas campestris* pv. *Vignicola* under *in vitro* condition (Jindal and Thind 1993). Considerable reduction of cotton bacterial blight could be obtained by using phylloplane bacterial antagonists isolated from cotton both under screen house and field conditions (Sanjay and Parashar, 2002). Bacterial blight of rice can be managed by plant growth promoting rhizobacteria (Islam and Bora, 1998) and plant associated strains of fluorescent pseudomonas capable of producing anti microbial antibiotic 2, 4 diacetyl phloroglucinol (Palaniyandi *et al*., 2006). Native isolate of fluorescent pseudomonas were capable of suppressing bacterial blight of cotton (Eddin *et al*. 2007). Guanghai *et al*. (2008) isolated a novel strain of *Lysobacter antibioticus* from rhizosphere of rice and found inhibitory to *X. oryzae* pv. *oryzae*

**2.4. Endophytes**

Endophytic bacteria are ubiquitous in most plant species and influence the host fitness, disease suppression, contaminant degradation and plant growth promotion. They colonize plant interior, interact more closely with host with less

competition for carbon sources and provide a more protected environment for fixation. Many authors have defined it and some of the relevant definitions are included .Perrotti (1926) defined endophytes as non pathogenic bacteria residing in plant tissue. According to Carroll (1986) endophytes are asymptomatic microorganisms living inside the plant. In 1991, Petrini defined it as a microorganism that inhabit at least for one period of their life cycle in host plant without causing any apparent harm. Kado (1992) identified it as bacteria that reside within plant tissues without doing substantial harm or gaining benefit other than securing residency. According to Quispel (1992), endophytic bacteria establish an endosymbiosis with plant, whereby plant receives an ecological benefit from the presence of the symbionts such as increased stress tolerance or plant growth promotion. Hallmann *et al*. (1997) described a bacterium as endophyte if it could be isolated from surface disinfected plant tissue or extracted from inside the plant if it did not visibly harm the plant. Azevedo and Araujo (2007), defined endophytes as all microorganisms, culturable or not, that inhabit the interior of plant tissue causing no harm to the host and that do not develop external structures, excluding in this way the nodulating bacteria and mycorhizal fungi.

**2.5. Occurrence and diversity of endophytes**

Perrotti (1926) first proposed the presence of non pathogenic bacteria in plant tissue. Now there are numerous reports of endophytic bacteria in seeds, ovules, root stem, leaves fruits and tubers without causing visible damage (Manjula *et al*., 2002). The genera *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Phyllobacterium*, *Pseudomonas* and *Serratia* have been reported as endophytes from several plants, but *Bacillus* and *Pseudomonas* are predominant ( Fisher *et al*., 1992).Mahaffee and Kloepper(1997) also reported the predominance of *Bacillus* sps. The diversity of bacterial genera was greater in roots than in stem (Hallmann *et al*., 1997).

**2.6 Isolation of endophytes**

Isolation of endophytes has key importance for research with endophytes (Hallmann *et al*., 1997). The most common method of isolation is trituration of surface disinfected plants using various disinfectants like Sodium hypochlorite (Fisher *et al*. 1992), Ethanol (Dong *et al*. 1994), Hydrogen peroxide, Mercuric chloride (Srikandarajah *et al* .1993) or a combination of two or more of these disinfectants (Pleban *et al*., 1995). According to Hallmann *et al*. (1997) the plant tissue is triturated with mortar and pestle in sterile water or buffer after surface disinfection and further this triturate is used for enumerating endophytes. They also suggested to include sterility checks to monitor the efficiency of disinfection procedure. Sterility check can be kept by transferring 0.1ml of final washing solution to test tube with bacterial growth medium (Mc lnroy and Kloepper, 1994) or imprinting the surface disinfected plant tissue on nutrient medium (Pleban *et al*. 1995). Hallmann *etal*. (1997) reported other methods of extractions like vacuum pressure extraction, centrifugation etc.

**2.7. Mode of action of endophytes**

Intensive work on biocontrol agents has shown that endophytic microorganisms isolated from surface disinfected plant tissue exhibit potential as biocontrol agent against microbial pathogens, insects and nematodes (Carroll, 1986). Chanway (1996) reported that endophytic bacteria use similar mechanism as epiphytic bacteria to suppress the deleterious microorganisms.

Endophytic bacteria inhibit growth of the pathogen by production of antimicrobial compounds (Leyns *et al*., 1990) siderophore (Kloepper *et al*., 1980) or by nutrient competition (Lockwood, 1990). They are capable of inducing systemic resistance and other defense responses (Vanpeer *et al* ., 1991) along with deposition of structural barriers in cell wall (Benhamou, 1998).

Bell *et al*. (1995) reported that competition for iron has been associated with biological control activity of certain endophytic bacteria. Bacterial endophytes prevent disease development through endophyte mediated de novo synthesis of structural compounds and fungi toxic metabolites which may result in certain forms of Systemic Acquired Resistance (SAR) (Sturz *et al*., 2000). Strong inhibition of mycelial growth, conidia formation, and germination of *Colletotrichum musae* by endophytic *Bacillus* strain was observed by He *et al*. 2002). Endophytic strain isolated from tobacco inhibited *Phytophthora nicotianae* by imparting direct and induced resistance, inhibiting mycelial growth, germination of zoospores and production of higher amount of defense related enzymes like Peroxidase and Phenyl Ammonia Lyase. An and Ma (2008) noticed that, spore germination and elongation of germ tube of grey mould, *Botrytis cinerea* was inhibited by endophytic strain isolated from cucumber. Rajendran *et al*. (2006) opined that bacterial endophytes promote plant growth and improve hosts capacity to withstand pathogen attack by competition, antibiosis and by inducing ISR. According to Azevedo and Araujo (2007), antagonistic endophytes colonize the specific site and inhibit the pathogen by competition or antibiosis. Among 103 endophytic bacterial strains isolated from cotton only two strains of *Bacillus* spp. significantly suppressed the disease by inhibiting mycelial growth (Rajendran and Samiyappan, 2008).

Endophytic bacteria enhanced plant growth by producing plant growth regulators such as IAA (Holland, 1997). Promotion of shoot height and fresh weight by endophytic bacteria isolated from red clover and potatoes was observed by Sturz *et al*., (1998) and in rape seed and tomato by Nejad and Johnson (2000). Sturz *et al*. (2000) reported that some plant endophytic bacterial association could increase plant growth and hasten development or improve resistance to environmental stress. Potential of endophytic bacteria isolated from soyabean in plant growth production was evaluated by Kulinsky *et al*. (2004). They observed that 34 percent of endophytic bacteria were able to produce IAA and 49 percent were able to solubilize mineral phosphate. Parihar *et al.* (2004) noticed that, out of

twelve endophytic bacteria isolated from sugarcane, nine showed phosphate solubilizing activity. Among five endophytes isolated from rice three were able to produce IAA (Tripathi *et al*., 2006). Kuss *et al*. (2007) reported that endophytic diazotrophic bacteria isolated from rice are capable of producing IAA. Some strains of endophytic bacteria isolated from rape and tomato are capable of producing volatile metabolites like HCN (Nejad and Johnson, 2000). Paul (2004) reported that *Pseudomonas fluorescens* isolated from black pepper were capable of producing siderophores and volatile compounds like HCN which help them to inhibit growth of the pathogen. Tripathi *et al*. (2006) isolated five endophytic bacteria from rice plants which were found as siderophore producers. Vijayaraghavan (2007) while studying the plant growth promoting rhizobacteria mediated ISR against bacterial wilt of ginger reported that PGPR isolates are capable of producing ammonia, siderophore, antibiotics and plant growth promoting hormones.

**2.8 Endophytes in plant disease management**

There are several reports from various crops showing the efficiency of endophytes in managing various plant diseases. Management of *Fusarium* wilt, *Verticillium* wilt and damping off of cotton were reported by many workers (Cubukcu and Benlioglu, 2007 and Rajendran and Samiyappan, 2008). Rahman and Khan (2002); Liu *et al*. (2003) and Muthukumar (2008) observed the control of wilt of solanaceous vegetables using endophytic microbes. Management of diseases of banana using endophytes was attempted by He *et al.* (2002) and Ting *et al.* (2008). Management of Quick wilt of pepper and basal stem rot of coconut using antagonistic endophytes were reported by Aravind *et al*. (2009) and Zaiton *et al*. (2008).

Several workers reported the efficiency of endophytes under field conditions. Endophytic bacteria isolated from ginger enhanced tillering, overall growth of plants and further suppressed the pathogen and disease incidence under field

condition (Rajan *et al*., 2000). Endophytic microbes isolated from amaranth were capable of suppressing amaranth leaf blight pathogen both under *in vitro* and *in vivo* conditions (Saisree, 2007). Management of chilli damping off caused by *Pythuim aphanidermatum* using bacterial endophytes were evaluated under glass house condition by Muthukumar (2008) and opinioned that, combined application of endophytes recorded lowest incidence of pre and post damping off.

**2.9. Compatibility of endophytes with plant protection chemicals**

Bhavani (2004) who studied the biological management of Phytophthora pod rot reported that Akonim 40, Indofil M 45 and Bavistin were compatible with bacterial antagonists to varying extends. The *in planta* studies with Pseudomonas strains confirmed compatibility with the fungicides tested *viz.*, Metalaxyl, Mancozeb, Potassium phosphonate and Carbendazim. Copper oxychloride was found incompatible with bacterial strain (Paul, 2004). Vijayaraghavan (2007) reported that PGPR was compatible with all tested fungicides except copper fungicides.

Mathew (2003) reported the compatibility of insecticides with *P. fluorescens*. He also reported that recommended dose of Imidachlorprid, Etofenprox, Chlorpyriphos and Triazophos were compatible with *P. fluorescens*. Bhavani (2004) reported that, the lower two concentration of insecticides *viz.,* Sevin, Ekalux, Nuvacron and Endosulfan were compatible with *Pseudomonas* strain compared to their higher concentrations. Phorate at all concentrations was found compatible. Vijayaraghavan (2007) studied the compatibility of PGPR with insecticides and reported that they were compatible with all insecticides tested.

Bhavani (2004) reported that, fertilizers *viz*., Rajphos and Muriate of Potash (MOP) were compatible compared to Urea which restricted the growth of antagonists. In addition, ammonium chloride and ammonium sulphate showed varying levels of inhibition showing their partial compatibility. Vijayaraghavan

(2007) reported that, all the fertilizers tested were found compatible with PGPR isolates. But they were found sensitive to antibiotics tested. Rangeshwaran and Kumar (2008) tested the antibiotic resistance of endophytes isolated from chick pea and compared them with rhizosphere bacteria. Five endophytic bacteria *viz., Erwinia herbicola*, *Enterobacter agglomerans*, *Bacillus megaterium*, *Bacillus* spp. and *Bacillus circulans* were tested for their antibiotic resistance along with rhizosphere bacteria *Pseudomonas fluorescens* and *Bacillus subtilis*. Endophytes seemed to be less resistant to antibiotics. *B. circulans* was highly susceptible to all antibiotics tested except Amoxicillin. *Enterobacter agglomerans*, *Bacillus megaterium*, *Bacillus* sp showed some resistance.

**2.9 Mutual compatibility of endophytes**

Manimala (2003) while studying the management of bacterial wilt of solanaceous vegetables using microbial antagonist reported that they are mutually compatible with each other and no lysis occurred at the juncture between the antagonists. The mutual compatibility of PGPR isolates with each other was reported by Vijayaraghavan (2007) while studying the plant growth promoting rhizobacteria mediated ISR against bacterial wilt of Ginger.

# *Materials and Methods*

# 3. MATERIALS AND METHODS

The present investigation on the "Potential of antagonistic endophytes against bacterial blight of anthurium" was carried out at the Department of Plant Pathology, College of Horticulture, Vellanikkara during 2006-2008

## 3.1 Isolation of the pathogen

The pathogen causing bacterial blight of anthurium was isolated from naturally infected plants collected from two locations *viz.,* Nelliyampathi area of Palakkad and Vellanikkara area of Thrissur districts. The infected leaf samples were washed thoroughly and subjected to ooze test. The diseased leaves with profuse ooze were selected, the infected areas were cut into small bits, surface sterilized with 1 per cent sodium hypochlorite solution for one minute. These bits were then washed in three changes of sterile water and teased apart to get a bacterial suspension. The suspension was streaked on Potato Sucrose Peptone Agar (PSPA) medium (Appendix I) to get well isolated colonies of the bacterium. The plates were incubated for 48 h at room temperature. Characteristic single colonies were selected on the basis of its colour, fluidity and slime and purified by repeated streaking on PSPA medium.

## 3.2 Pathogenicity test

A thick suspension of 24 h old culture of the isolate was inoculated on leaves of anthuruim by giving pin prick. The culture was smeared on both surfaces of leaves by means of cotton dipped in bacterial suspension. The inoculated plants were covered with polythene cover and kept under shade. The pathogen was re-isolated from artificially inoculated plants. Single colonies of such isolates were compared with original isolate for their typical morphological characters.

### 3.3 Symptomatology

Symptoms produced by the pathogen on the anthurium plants both under natural and artificial conditions were studied in detail.

### 3.4 Characterization and identification of the pathogen

The cultural, morphological, physiological and biochemical characters of the pathogen were studied based on methods recommended in Laboratory Methods in Microbiology (Harrigan and Mc Cane, 1966) for appropriate identification. For each test 24-48 h old culture of the pathogen was used.

#### 3.4.1 Cultural characters

The colony morphology was studied from 24-48h old culture of the bacterium grown on PSPA medium. Colonies were observed for their colour, shape, size, elevation, margin and fluidity.

#### 3.4.2 Pigment production

Production of water insoluble and water soluble pigments by the isolate was studied by streaking on Yeast Glucose Chalk Agar (Appendix I) and King's B medium (Appendix I).

#### 3.4.3 Morphological characters

##### 3.4.3.1 Gram's reaction

For Gram staining 24 h old bacterial cultures was used. Shape of bacteria was observed under oil immersion objective. To confirm the result of Grams reaction, KOH test was also conducted.

#### 3.4.3.2 Solubility in 3% KOH

A loopful of bacterial culture was placed on a clear glass slide. One drop of three per cent KOH solution was added over it, thoroughly mixed with the help of inoculation needle and lifted up and down to know solubility in KOH.

#### 3.4.3.3 Endospore staining

A loopful of bacterial culture was taken and smeared on glass slide and heat fixed, then a few drops of 1.5 per cent amidoblack was added and allowed to stay for 70 sec. Then, the slide was washed under gentle stream of running water, stained for 20 sec with 1 per cent carbol fuschin and washed thoroughly under tap water. Then the slide was blot dried and observed under microscope for endospore.

### 3.4.4 Physiological characters

#### 3.4.4.1 Mode of utilization of glucose

To determine whether the bacterium utilized glucose only under aerobic condition or both under aerobic and anaerobic condition one per cent glucose was added to the prepared basal medium (Appendix I) and dispensed in tubes upto 4 cm. The medium was sterilized by tyndalization and inoculated in duplicate by stabbing with straight inoculation needle charged with bacterial growth. In one of the tubes, the medium was sealed with 1 cm layer of sterilized liquid paraffin. The tubes were incubated at room temperature and observations on change in colour were taken at regular interval upto 15 days.

#### 3.4.4.2 Citrate utilization test

One day old culture was streaked on the surface of Simmon's Citrate Agar (Appendix I) and observed for any colour change of the medium.

#### 3.4.4.3 Starch hydrolysis

The ability of the bacterium to hydrolyze starch was tested using Nutrient Agar Medium (Appendix I) containing 0.2 per cent soluble starch. Test organism was spotted on Petri plates containing medium. The dishes were flooded with Lugol's iodine solution after 48h of incubation. A colourless zone around the bacterial growth indicated positive starch hydrolysis compared to the blue background of the medium.

#### 3.4.4.4 Catalase test

Smear of one day old culture grown in PSPA medium was prepared on clear glass slide and covered with few drops of three per cent $H_2O_2$ and observed for the formation of effervescence.

#### 3.4.4.5 Denitrification test

Bacterial culture was stab inoculated into the Vanden Mooter Succinate Medium (Appendix I) and sealed with three ml of one percent molten agar and examined daily for production of gas under the seal.

#### 3.4.4.6 Oxidase test

The 24h old bacterial culture was spot inoculated on oxidase disc and the change in colour of the disc from white to purple or blue within 60 seconds was observed.

**3.4.4.7 Arginine dihydrolase reaction**

The bacterial cultures was stab inoculated into the semisolid medium of Thornley (1960) (Appendix I) and the tubes were incubated at room temperature for seven days and observed for colour change.

**3.4.4.8 Production of Hydrogen Sulphide**

The ability of bacterium to liberate hydrogen sulphide was tested using peptone water medium (Appendix I). Five ml of medium was dispensed in test tubes and autoclaved. Lead acetate paper strips of 5 x 50 mm size were prepared by soaking them in super saturated solution of lead acetate. The strips were dried autoclaved and again dried. The tubes were inoculated in triplicates with bacterial isolates and the lead acetate strips were inserted aseptically by the side of the plug in the tube. The tubes were incubated at room temperature and observations were recorded at regular intervals up to 14 days for blackening of test strip.

**3.4.4.9 Methyl Red Test**

Five ml of methyl red broth medium (Appendix I) was dispensed in tubes and sterilized by steaming for 30 seconds for three successive days. Tubes were then inoculated with 48 h old culture of bacterial isolate. The tubes were incubated for seven days at room temperature. Few drops of 0.02 per cent Methyl red in 50 per cent alcohol was added to culture tube. A distinct red colour indicated methyl red reaction

**3.4.4.10 Gelatin liquefaction**

Nutrient gelatin medium (Appendix I) was sterilized by autoclaving and cooled to 45 $^{0}$C. It was then poured into Petri plates and allowed to solidify. The medium was spot inoculated by 48h old culture of bacterium. After incubation of

two to seven days agar surface was flooded with 0.2 percent $HgCl_2$ solution in dilute HCl. A clear zone around the bacterial growth indicated liquefaction of gelatin.

**3.4.4.11 Production of Indole**

Tryptophan broth medium (Appendix I) was used for this test. The medium was dispersed in tubes and autoclaved .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 the strips get covered with oxalic acid, they were dried at room temperature and used without sterilization. The tubes were incubated with bacterial isolate 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 strip to pink or red indicates indole production.

**3.4.4.12 Growth on 6 % NaCl**

Peptone water with six percent NaCl was used for the test. The medium was dispensed in tubes; autoclaved and inoculated with bacterium and incubated. The ability of bacterium to grow on medium was observed.

**3.4.4.13 Lipolytic activity**

Sierra's medium (Appendix I) was employed for this test. The medium was dispensed in 99 ml quantities in flasks, autoclaved and cooled to $45^0$C. One ml of Tween 80 was added to the medium and thoroughly mixed. The medium was poured in sterile Petri dishes and test bacterium was spot inoculated on the medium. The plates were incubated and observed at regular intervals for 15 days. Opaque zone around the bacterial growth indicate positive lipase production.

### 3.4.4.14 Utilization of Carbon sources

Basal medium for Xanthomonads was supplemented with 1 per cent concentrated solution of carbon compound to be tested. 0.7 ml of five percent alcoholic solution of bromocresol purple was added to get reddish violet colour. Following carbon compounds *viz*, dextrose, fructose ,sucrose, glucose, maltose lactose mannose ,cellobiose,arabinose , adinitol,inositol,glycerol,mannitol,dulcitol and sorbitol were used for the test. Medium was sterilized by tyndalisation and slants were inoculated with bacterium, incubated at room temperature. The change in colour of medium from reddish violet to yellow indicated the production of acid.

### 3.4.4.15 Production of Ammonia

Peptone water was used for this test. The culture was inoculated in peptone water and incubated for 48 h. The accumulation of ammonia is detected by Nessler's reagent which gives brown to yellow precipitate with ammonia.

## 3.5 Isolation and enumeration of endophytic microbes

Endophytic microorganisms were isolated from stem, leaves and roots of healthy anthurium plants. For this, plants were collected from anthurium growing areas of Thrissur, Kannur, Kasargod and Thiruvananthapuram districts. Stem ,leaf and root samples were washed in tap water, weighed separately (1 g each) and then surface sterilized with one per cent Sodium hypochlorite solution for three minutes, followed by four changes in 0.02 M sterile potassium phosphate buffer (pH 7) .

Following surface disinfection, the samples were triturated with mortar and pestle in 9 ml of buffer. From this serial dilutions upto $10^{-2}$ of the triturate were made in potassium phosphates buffer. From each dilution one ml was

transferred to Petri plate. Three replications were maintained for each dilution and plates were incubated at room temperature. To decrease the possibility of recovery of surface contaminants, sterility checks were also maintained by transferring 0.1 ml of final buffer wash to Petri dishes containing solidified NA and PDA. Bacterial colonies developing after 24 h and that of fungal colonies after 48 h were selected and transferred to respective media. Thus 37 bacterial isolate and 14 fungal isolates were maintained for further studies.

### 3.6 *In vitro* evaluation of endophytes for their antagonistic effect against the pathogen

The antagonistic effect of isolated bacterial and fungal endophytes against *Xanthomonas axonopodis* pv. *dieffenbachiae* was studied under *in vitro* condition by adopting dual culture method (Dennis and Webster, 1971). Standard cultures of *Pseudomonas fluorescens* (KAU), *Trichoderma viride* (KAU), *T. harzianum* (IISR) were also used for comparison.

#### 3.6.1 Preliminary screening of bacterial endophytes

For preliminary screening, Nutrient Agar (NA) plate seeded with 48 h old culture of pathogen in Petri plate was spot inoculated with endophytic bacterial isolate. In each plate four different bacterial isolates were inoculated at equidistant points two cm away from the periphery of the plate.

The plates were incubated at room temperature and observed for inhibition of the pathogen after 48 h. The plates with pathogen alone served as control. Eight isolates which showed zone of inhibition were selected for further studies.

The bacterial isolates which showed antagonism in preliminary screening were further tested individually. These selected antagonists were again spot inoculated at the center of NA plate seeded with pathogen. The plates were

incubated at room temperature and observed for inhibition of the pathogen after 48 h. The plates with pathogen alone served as control. Inhibition zone was measured and per cent inhibition calculated.

### 3.6.2 Preliminary screening of fungal endophytes

A total of 14 fungal endophytes were tested for their antagonistic property against the pathogen. Agar blocks of 10mm diameter containing seven day old growth of fungal endophyte were inoculated at equidistant points, two cm away from periphery of the NA plate seeded with 48h old culture of pathogen. The plates were incubated at room temperature and observed for inhibition of pathogen after 48h. The plates with pathogen alone served as control. Only two isolates which showed zone of inhibition of pathogen were selected for further studies.

The fungal isolates which showed antagonism in preliminary screening were tested individually by spot inoculating at centre of NA plate seeded with pathogen and incubated at room temperature. The plates with pathogen alone served as control. Inhibition zone was measured and per cent inhibition was calculated.

### 3.7 Selection of antagonistic endophytes

Since fungal endophytes did not show much inhibition, only eight bacterial endophytes were selected for further studies in comparison with standard *P. fluorescens* isolate from KAU.

### 3.8 Mechanism of antagonism

Selected antagonist where further subjected to various tests for understanding the parameters that may contribute to disease control.

### 3.8.1 Vigour Index

The selected endophytes along with reference culture of *P. fluorescens* (KAU) were bio assayed for their ability to promote seedling growth using the method suggested by Shende *et al*. (1977) and Elliot and Lynch (1984) with few modifications. Sorghum seeds were surface sterilized with 0.1 per cent mercuric chloride for three minutes followed by successive washing with sterile water. Water was decanted and the seeds were soaked for 30 min in 48 h old cultures grown in nutrient broth, after which medium was decanted. Sterile filter paper discs soaked in sterile water was placed in the Petri dish. Soaked seeds were placed equidistantly on the filter paper and incubated for 72 h.Three replications were maintained and the seeds treated with sterile nutrient broth alone served as control. Germination percentage, length of epicotyl and hypcotyl were measured after 72 h.The Vigour Index (VI) was calculated using the formula:

VI = (Mean root length + Mean shoot length) x Germination percentage.

The endophytes were scored based on vigour index (VI) was as follows

VI of >1<2 = 1; VI of >2<3 = 2; VI of >3<4 = 3; VI of >4 = 4

### 3.8.2 Production of HCN

Production of HCN by isolates was tested by following method of Wei *et al*. (1991). Log phase of bacterial culture was inoculated to 25ml of Kings'B broth (Appendix I) supplemented with 4.4 $g^{-1}$ of glycine taken in a sterile Petri plate. Sterile filter paper strips soaked in picric acid solution was placed in the lid of each plate. Petri plates were sealed with paraffin and incubated for 72 h. Change in colour of filter paper from yellow to brown indicates production of HCN.

### 3.8.3 Production of ammonia

The selected bacterial endophytes were grown in 25ml of peptone water and incubated at $30^0$C for 4 days. Three replications were maintained for each isolate. After incubation 1 ml of Nessler's reagent was added to the broth. Presence of faint yellow or deep yellow or brown colour of the broth culture from yellow or brown indicates production of ammonia.

### 3.8.4 P solubilization

The P solublization capacity of bacterial endophytes was tested *in vitro* using Pikovskaya's agar medium (Appendix I). Ten ml of log phase of isolates were spot inoculated at centre of plate containing medium and incubated at $28^0$ C for five days. Plates were observed for clearing zone around colony and its diameter measured. Three replications were maintained for each isolate.

### 3.8.5 Qualitative estimation of IAA

IAA production was estimated using modified protocol suggested by Bric *et al.* (1991). A loop full of bacterial culture was inoculated in 25ml of Luria Bertini medium (Appendix I) and incubated for 24 h, at $28^0$C in rotary shaker. After 24h the broth cultures were centrifuged at 10,000 rpm for 15 minutes. Two ml of supernatant was taken and 2 drops of Orthophosphoric and 2 ml of Salkowsky's reagent (1ml of 0.5M $Fecl_3$ in 50ml of 35 percent $HClO_4$) was added and incubated at $28^0$ C for 30 minutes and absorbance was measured at 530 nm. A standard curve was prepared with different concentrations of IAA and was used to quantify the IAA production.

### 3.9 Detection of siderophores

Siderphore production was detected by UV fluorescence method. Log phase of the endophyte cultures including reference culture were streaked on King's B (KB) plate and incubated at $28^0$C for 48h. The plates were observed on UV trans-illuminator to view the fluorescence (Kloepper *et al.,* 1980)

Iron dependent production of siderophores was also estimated. The King's B medium was amended with two concentrations of $FeCl_3.6H_2O$ @ 1 and 10 mg $l^{-1}$. The sterilized medium was poured into each Petri plate. The test cultures were streaked on the surface of the medium. Three replications were maintained. The inoculated plates were incubated at room temperature for 48h. Observations on production of greenish yellow fluorescent pigment were recorded.

### 3.10 Compatability of selected endophytes to plant protection chemicals and plant nutrients

The compatibility of selected endophytes and standard culture of *Pseudomonas fluorescens* to antibiotics, fungicides, insecticides and fertilizers were studied by Filter paper disc method.

### 3.10.1 Antibiotics

Antibiotic sensitivity was assessed by Hi-media antibiotic discs. The following antibiotics at various concentrations used for *in vitro* evaluation are given below.

| Sl. No. | Antibiotic | Concentration (ppm) |
|---|---|---|
| **1** | **Chloramphenicol** | **0.1, 0.25, 0.5** |
| **2** | **Rifampicin** | **0.05, 0.15, 0.3** |
| **3** | **Gentamycin** | **0.3, 0.5** |
| **4** | **Ampicillin** | **0.1, 0.25** |
| **5** | **Streptomycin** | **0.1, 0.25** |
| **6** | **Tetracycline** | **0.1, 0.3** |
| **7** | **Pencillin-G** | **0.1** |

The effect of commercially available Streptocycline and Turmeric sodium bicarbonate mixture was also tested. Concentrations used for the evaluation are given below.

| Sl. No | Chemical name | Concentration (ppm) |
|---|---|---|
| **1** | **Streptocycline** | **200, 300, 400** |
| **2** | **Turmeric-sodium bicarbonate mixture(10:1)** | **500, 1000, 1500** |

### 3.10.2 Fungicides

The following fungicides with different concentrations were used for compatibility study. The details are given below.

| Sl. No. | Chemical name | Trade name | Concentration (%) |
|---|---|---|---|
| **1** | **Mancozeb** | **Indofil M-45** | **0.2, 0.3, 0.4** |
| **2** | **Captan** | **Captaf** | **0.2, 0.3, 0.4** |
| **3** | **Carbendazim** | **Bavistin** | **0.05, 0.1, 0.2** |
| **4** | **Potassium phosphonate** | **Akomin-40** | **0.2, 0.3, 0.4** |
| **5** | **Hexaconazole** | **Contaf** | **0.05, 0.1, 0.2** |
| **6** | **Mancozeb + Carbendazim (63%) (12%)** | **Saaf** | **0.2, 0.3, 0.4** |

### 3.10.3 Insecticides

The compatibility of the isolates with insecticides at different concentrations as shown below was studied.

| SI. No. | Chemical name | Trade name | Concentration (per cent) |
|---|---|---|---|
| **1** | **Chlorpyriphos** | **Classic 20 EC** | **0.05, 0.1, 0.15** |
| **2** | **Dimethoate** | **Rogor 30 EC** | **0.05,0.1,0.15** |
| **3** | **Quinalphos** | **Ekalux 25 EC** | **0.05, 0.1, 0.2** |
| **4** | **Malathion** | **Malathion 30 EC** | **0.05, 0.1, 0.15** |
| **5** | **Monocrotophos** | **Target 36 SL** | **0.1, 0.125, 0.15** |
| **6** | **Triazophos** | **Hostathion 40Ec** | **0.1, 0.125, 0.15** |

### 3.10.4 Fertilizers

Different fertilizers and their concentrations used for evaluation of compatibility are given below.

| Sl. No. | Name | Concentration (%) |
|---|---|---|
| **1** | **Urea** | **1.0, 1.5, 2.0** |
| **2** | **Rajphos** | **2.0, 2.5, 3.0** |
| **3** | **Muriate of potash (MOP)** | **2.0, 2.5, 3.0** |
| **4** | **Factomphos** | **2.0, 2.5, 3.0** |

Antibiotic sensitivity was assessed by Hi-Media antibiotic discs. In case of Streptocycline, turmeric-sodium bicarbonate mixture, fungicides, insecticides and fertilizers in order to get desired concentration the required quantity was added to 100 ml sterile water and autoclaved filter paper disc of 10mm diameter were soaked in the various concentrations of plant protection chemicals /fertilizers for a period of 30 seconds. Then filter paper discs were placed on PSPA medium seeded with endophyte. Control consists of medium alone with antagonists. Three replications were maintained and incubated at room temperature. Observations on inhibition zone around disc were taken 48 h after incubation and the per cent inhibition over control was calculated.

### 3.10.5 Compatibility of pathogen to plant protection chemicals

Streptocycline, Captaf, Mancozeb and turmeric-sodium bicarbonate mixture were used for *in vitro* testing following filter paper disc method. The chemical and their concentrations are given below. Observations on zone of inhibition were recorded after 48 h of incubation and per cent inhibition over control was calculated.

| Sl. No. | Chemical name | Concentration (ppm) |
|---|---|---|
| **1** | **Streptocycline** | **200, 300, 400** |
| **2** | **Mancozeb** | **2000, 3000, 4000** |
| **3** | **Captaf** | **500, 1000, 1500** |
| **4** | **Turmeric-sodium bicarbonate mixture** | **500, 1000, 1500** |

### 3.11 Mutual compatibility of selected endophytes

To assess the mutual compatibility of the eight selected endophytes including reference culture of *P. fluorescens*, the organisms were streaked perpendicular to each other on plates containing NA medium (Manimala, 2003). Three replications were maintained. The plates were incubated for 48 h at room temperature and observed for any lysis at the juncture between the antagonists.

### 3.12 Evaluation of potential endophytes against bacterial blight pathogen

Based on the *in vitro* antagonism and the mechanisms studied five endophytes were selected. A pot culture experiment was laid out to study the antagonistic efficacy of the five selected endophytes in comparison with reference culture of *P. fluorescens* (KAU), Turmeric-sodium bicarbonate mixture and Streptocycline in reducing bacterial blight incidence and severity. The experiment was carried out during July-September 2007 at College of Horticulture, Vellanikkara.

**Details of experiment are as follows**

Design : CRD
Treatments : 9
Replications : 5
Variety : Tropical red
Method of application : Foliar spray

The treatment details of the experiment is given below

| Sl. No. | Treatments | Isolates/chemicals |
|---|---|---|
| **1** | **$T_1$** | **EB-12** |
| **2** | **$T_2$** | **EB-13** |
| **3** | **$T_3$** | **EB-15** |
| **4** | **$T_4$** | **EB-26** |
| **5** | **$T_5$** | **EB-31** |
| **6** | **$T_6$** | ***P. fluorescens* ( KAU)** |
| **7** | **$T_7$** | **Streptocycline (300ppm)** |
| **8** | **$T_8$** | **Turmeric + sodium bicarbonate mixture (10:1)** |
| **9** | **$T_9$** | **Control** |

EB: Endophytic bacteria

### 3.10.1 Preparation of bacterial inoculum for application

The five endophytic bacteria and standard culture of *P. fluorescens* (KAU) were grown separately on NA medium for 48 h. The bacterial inoculum was prepared in sterile distilled water and concentration was adjusted to $10^8$ cfu $ml^{-1}$.

### 3.10.2 Preparation of turmeric-sodium bicarbonate mixture

Turmeric-sodium bicarbonate mixture is prepared by mixing ten parts of turmeric with one part of sodium bicarbonate. 0.15 per cent solution was used for spraying.

### 3.10.3 Foliar Spray

Application of selected bacterial endophytes, biocontrol agent, turmeric- sodium bicarbonate mixture and antibiotic were given as two pre-inoculation and two post-inoculation sprays at five days intervals

### 3.10.4 Artificial inoculation of the Pathogen

A thick suspension of 24-48h old culture of bacterium was inoculated on leaves of anthurium plant by smearing the inoculam on both surface of leaf lamina by cotton dipped in the suspension after giving pinpricks on leaves.

### 3.11 Observations

Observations on disease incidence and disease severity were recorded based on the score chart (Dhanya, 2000) on the day of spraying and five days after each spraying upto one month.

### 3.11.1 Assessment of disease incidence

For assessing the disease incidence, number of infected leaves and total number of leaves infected in each treatment was recorded .Per cent disease incidence was calculated using the formula.

$$\text{Per cent disease incidence (PDI)} = \frac{\text{Number of leaves infected}}{\text{Total number of leaves observed}} \times 100$$

### 3.11.2 Assessment of Per cent disease severity

For assessing disease severity, diseased leaves were numerically rated and maximum disease grade was recorded based on score chart designed by (Dhanya,

2000) and Per cent disease severity was calculated using formula suggested by Wheeler (1969)

**Table .3.1 Score chart of bacterial blight disease of anthurium**

| Grade | Infection (%) | Description |
|---|---|---|
| **0** | **0** | **No lesion** |
| **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 leaves with blackening of petioles of leaves** |
| **5** | **76-100** | **Complete death of the plant** |

$$PDS = \frac{\text{Sum of numerical ratings}}{\text{Total number of leaves observed x Maximum disease grade}} \times 100$$

### 3.12 Characterization of endophytes

Characterization was carried out by following methods adopted for characterization of the pathogen. The cultural, morphological and physiological characters were studied in detail.

### 3.13 Statistical analysis

Analysis of variance was performed on the data collected in various experiments using statistical package MSTAT (Freed, 1986). Multiple comparison among treatment means was done using DMRT.

# *RESULTS*

# 4. RESULTS

Investigations on the topic "Potential of antagonistic endophytes against bacterial blight of anthurium" were carried out at the Department of Plant Pathology, College of Horticulture, Vellanikkara during 2006-2008 and the results are presented below:

## 4.1 Isolation of the pathogen

The bacterial blight pathogen was isolated from infected anthurium leaves on Potato Sucrose Peptone Agar (PSPA) medium. Isolation of the bacteria on PSPA medium yielded yellow, circular, slimy, convex and smooth colonies with entire margin. The isolate was purified by dispensing a loop full of culture in sterile water and by repeated streaking on PSPA medium. The culture was preserved both in slants as well as in sterile water and stored under refrigerated condition for further studies.

## 4.2 Pathogenicity test

Pathogenicity of the organism was established by swabbing the culture on pinpricked leaves of healthy anthurium plants. Re-isolation from such infected plants yielded colonies resembling original isolate of the bacterium.

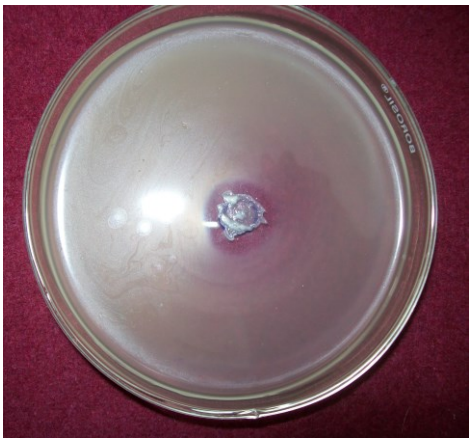
## 4.3 Symptomatology

The symptomatology of disease was studied in detail under natural and artificial condition. Naturally infected plants showed a variety of symptoms. The initial symptom of the disease appeared as irregular water soaked lesion on leaf margin and lamina (Plate 4.1). It increased in size and turned dark brown surrounded by prominent yellow halo. When considerable portion of lamina got blighted, those leaves were shed. Symptoms were also present on spathe as well

**Plate 4.1 Symptoms of bacterial blight of anthurium**

**Plate 4.2 Inhibition zone produced by endophytes EB-15 and EB-31 on the pathogen**

EB-15



EB-31

**Table: 4.1 Cultural, morphological and biochemical characters of the pathogen**

| Sl. No. | Cultural, morphological & biochemical characters | Reaction of pathogen | Sl. No. | Cultural, morphological & biochemical characters | Reaction of pathogen |
|---|---|---|---|---|---|
| **1** | **Grams reaction** | **-ve** | **17** | **Utilization of carbon compounds with acid production** | |
| **2** | **Margin** | **Entire** | **a** | **a.Glucose** | **+ve** |
| **3** | **Surface** | **Small smooth** | **b** | **b.Maltose** | **+ve** |
| **4** | **Configuration** | **Rod** | **c** | **c. Lactose** | **+ve** |
| **5** | **Pigment production** | | **d** | **d. Fructose** | **+ve** |
| **a** | **Water soluble** | **-ve** | **e** | **e. Dextrose** | **+ve** |
| **b** | **Non water soluble** | **+ve** | **f** | **Sucrose** | **+ve** |
| **6** | **Mode of utilization of glucose** | | **g** | **Mannose** | **+ve** |
| **a** | **Aerobic** | **+ ve** | **h** | **Arabinose** | **+ve** |
| **b** | **Anaerobic** | **- ve** | **i** | **Sorbitol** | **-ve** |
| **7** | **Citrate utilization test** | **+ve** | **j** | **Inositol** | **-ve** |
| **8** | **Starch hydrolysis** | **+ve** | **k** | **Cellobiose** | **-ve** |
| **9** | **Production of $H_2S$** | **+ve** | **l** | **Adinitol** | **-ve** |
| **10** | **MR test** | **-ve** | **m** | **Glycerol** | **-ve** |
| **11** | **Gelatin liquefaction** | **+ve** | **n** | **Dulcitol** | **-ve** |
| **12** | **Production of indole** | **-ve** | **o** | **Mannitol** | **-ve** |
| **13** | **Nitrate reduction** | **-ve** | **18** | **Ammonia production** | **+ ve** |
| **14** | **Catalase test** | **+ve** | **19** | **Urease test** | **-ve** |
| **15** | **Growth in 6% NaCl** | **-ve** | **20.** | **Arginine dihydrolase** | **+ve** |
| **16** | **Lipolytic activity** | **+ve** | | | |

+ve: Positive, -ve: Negative

as on spadix. On spathe, instead of a yellow halo, brownish black lesions with wavy fringed margin were formed. Affected spadix finally turned to black candle like structure. Systemic infection started as water soaked lesion at collar region of plant leading to rotting. Leaves showed browning and yellowing. Infection spread from collar region through the petiole to the leaf base and to the leaf lamina. In about two to three weeks after the appearance of first symptoms, the whole plant dried up.

Under artificial inoculation also, symptom appeared as minute water soaked lesions on leaf lamina and margin. It later increased in size and was surrounded by chlorotic halo. When considerable portion of lamina got blighted, those leaves were shed and finally entire plant was killed.

**4.4 Characterization and identification of the pathogen.**

The bacterium produced yellow, circular, slimy, convex colonies with entire margin on Potato Sucrose Peptone Agar (PSPA) medium. Gram staining revealed that the isolate was Gram negative short rods (Table 4.1). The bacterium was found to be aerobic and positive to catalase test. Isolate utilized citrate as carbon source. It produced ammonia, $H_2S$ and non-water soluble yellow pigment . It did not produce nitrite from nitrate and was negative to urease test, indole production and Methyl Red (MR) test. The bacterium was unable to grow on media containing 6 per cent NaCl. It was found to be positive to Arginine dihydrolase test and test for lipolytic activity. It liquefied gelatin, hydrolyzed starch and produced ammonia. Out of 15 sugars tested, the isolate utilized glucose, maltose, lactose, dextrose, fructose, sucrose, mannose and arabinose and not the others.

### 4.5. Isolation and enumeration of endophytes

Endophytic microbes were isolated from different parts of healthy anthurium plants collected from various locations of Thrissur, Kannur, Kasargod and Thiruvananthapuram districts and the results are presented in the Table 4.2. From the data, it is evident that the population of endophytic microorganisms varied with different locations. Among the endophytes, bacteria were more in number than fungi. The population of fungi showed variation among different samples. Highest count of fungi (2.67 x $10^2$ cfu $g^{-1}$) was noticed in root samples collected from Thrissur. Lowest count of fungi (0.67 x $10^2$ cfu $g^{-1}$) was noticed in petiole sample collected from Kasargod whereas bacterial population was the highest (7.33 x $10^2$ cfu $g^{-1}$) in root sample collected from Thiruvanathapuram and the least count (1.33 x $10^2$ cfu g-1) in petiole and leaf samples collected from Kasargod. Based on cultural characters, 51 representative colonies were sub cultured and used for further studies. These included 14 fungal and 37 bacterial endophytes.

### 4.6 *In vitro* evaluation of endophytes for their antagonistic effect against the pathogen

The antagonistic effect of isolated bacterial and fungal endophytes against *Xanthomonas axonopodis* pv. *dieffenbachiae* was studied under *in vitro* condition by adopting dual culture method (Dennis and Webster 1971). Standard cultures of *Pseudomonas fluorescens* (KAU), *Trichoderma viride* (KAU) and *T. harzianum* (IISR) were also used for comparison.

#### 4.6.1 Preliminary screening of bacterial endophytes

The antagonism of bacterial endophytes against bacterial blight pathogen *Xanthomonas axonopodis* pv. *dieffenbachiae* was tested in comparison with the standard culture of P.flurescence (kau) bioagent on Nutrient Agar

**Table 4.2 Quantitative estimation of endophytic microorganisms of anthurium**

| Sl. No. | Location | Part used | Fungi* ($x10^2$ cfu/g) | Bacteria* ($x10^2$ cfu/g) |
|---|---|---|---|---|
| **1** | **Thiruvananthapuram** | **Root** | **1.67** | **7.33** |
| | | **Petiole** | **1.33** | **3.67** |
| | | **Leaf** | **1.67** | **4.33** |
| **2.** | **Thrissur** | **Root** | **2.67** | **5.67** |
| | | **Petiole** | **1.67** | **2.67** |
| | | **Leaf** | **2.33** | **3.33** |
| **3.** | **Kannur** | **Root** | **1.67** | **3.67** |
| | | **Petiole** | **1.33** | **2.33** |
| | | **Leaf** | **1.33** | **3.33** |
| **4.** | **Kasargod** | **Root** | **1.67** | **3.67** |
| | | **Petiole** | **0.67** | **1.33** |
| | | **Leaf** | **1.33** | **1.67** |

* Average of three replications

**Fig.4. 1: *In vitro* antagonistic effect of endophytes against the pathogen**

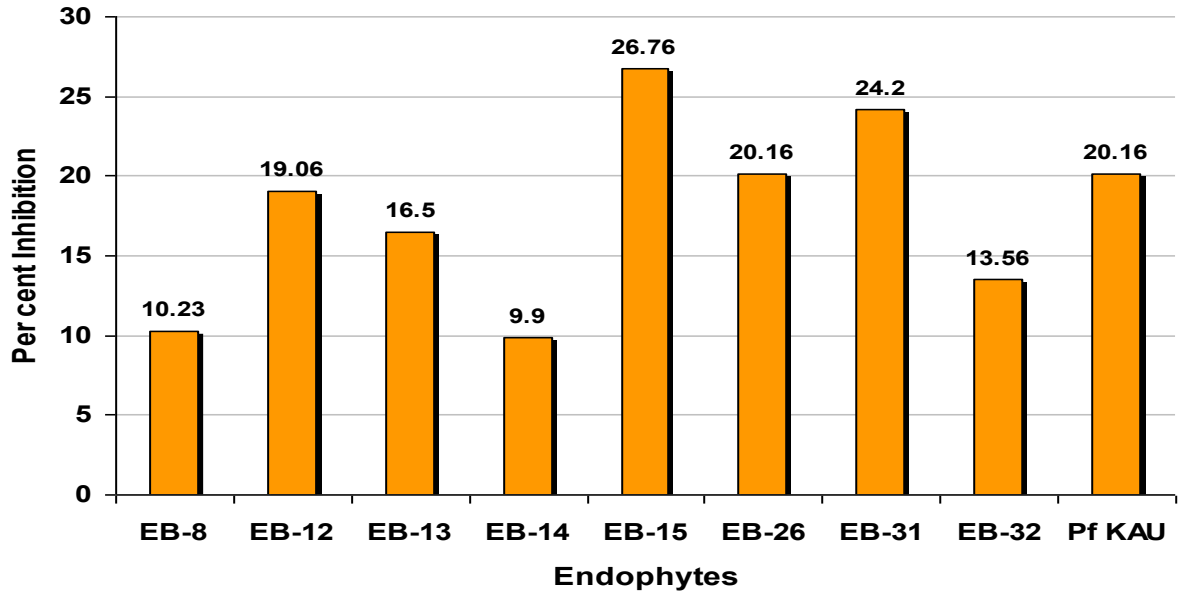



**Fig.4.2: Production of Indole Acetic Acid by selected endophytes of anthurium**

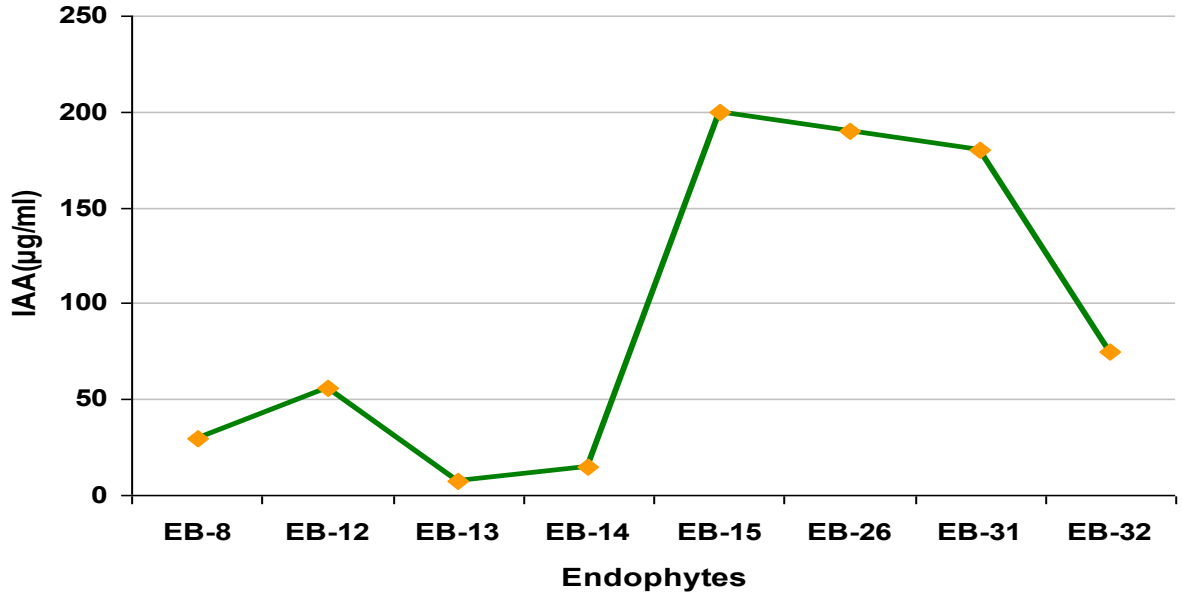


**Table 4.3 *In vitro* inhibitory effect of bacterial endophytes against the pathogen**

| Sl. No. | Endophytes | *Diameter of inhibition zone (mm) | Per cent inhibition over control | ***Score |
|---|---|---|---|---|
| 1 | EB-8 | 9.3 | 10.23[g] | 1 |
| 2 | EB-12 | 17.3 | 19.06[d] | 2 |
| 3 | EB- 13 | 15.0 | 16.50[e] | 2 |
| 4 | EB-14 | 9.0 | 9.9[g] | 1 |
| 5 | EB-15 | 24.3 | 26.76[a] | 3 |
| 6 | EB-26 | 18.3 | 20.16[c] | 2 |
| 7 | EB-31 | 22.0 | 24.20[b] | 3 |
| 8 | EB-32 | 12.3 | 13.56[f] | 2 |
| 9 | **_Pf_ KAU | 18.3 | 20.16[c] | 2 |

* Mean of three replications

** _Pf_1: _Pseudomonas fluorescens_ (KAU)

*** Score: inhibition zone >1<10mm = 1, >10<20mm = 2, >20<30mm = 3, >30 = 4

plates by dual culture method. Out of 37 bacteria screened, eight were found to be antagonistic to the pathogen as evidenced by varying size of zone of inhibition.

The bacterial isolates which showed antagonism in preliminary screening were further tested individually. The inhibition zone produced by standard culture of *P. fluorescens* (KAU) was also studied. All the isolates tested were antagonistic to the *X.axonopodis* pv. *dieffenbachiae* showing per cent inhibition ranging from 9.9-26.76 mm.(Fig 4.1). From the results in Table (4.3) it is clear that isolates EB-15, EB-26 and EB-31 showed more than 20 per cent inhibition. The reference culture of P. *fluorescens* (KAU) was equally effective against the pathogen. EB-15 showed maximum per cent inhibition (26.76) followed by EB-31 (24.20) (Plate 4.2). EB-14 recorded the least value (9.9).

### 4.6.2 Preliminary screening of fungal endophytes

The antagonism of fungal endophytes against bacterial blight pathogen *Xanthomonas axonopodis* pv. *dieffenbachiae* was tested in comparison with the standard cultures of fungal (*T. harzianu*m and *T. viride*) bioagents on Nutrient Agar plates by dual culture method. Out of 14 fungi screened, only two showed slight zone of inhibition

### 4.7 Selection of antagonistic endophytes

Since fungal endophytes did not show much inhibition, only eight bacterial endophytes were selected for further studies in comparison with standard *P. fluorescens* isolate from KAU.

### 4.8 Mechanism of antagonism

The selected antagonists were further subjected to various tests for understanding the parameters that contributed to their inhibitory action against the pathogen.

#### 4.8.1. Vigour Index

The eight endophytes along with reference culture of *P. fluorescens* (KAU) were bioassayed for their ability to promote seedling growth. The highest germination per cent of 96.67 was observed in sorghum seeds treated with EB-15 (Table 4.4). Maximum shoot length of 5.12 cm was with seeds bacterized with EB-31 followed by EB-15 and EB-8 . Sorghum seeds bacterized with EB-15 were found to be superior plant growth activator as it showed the highest vigour index (VI) 7.39 closely followed by EB-31, EB-8, *P. fluorescens* (KAU). The least vigour index of 3.10 was observed in control followed by EB-32. It was noticed that except for plants in control and EB-32 all others showed a vigour index score of four.

#### 4.8.2 Production of HCN

Performance of the selected endophytic bacteria in the production of volatile metabolites was tested on Kings B broth supplemented with glycine. Following 72 h incubation, most of the isolates were found to be HCN negative. Only two of the isolates *viz.,* EB-14 and EB-31 showed detectable cyanide production by changing the colour of filter paper from yellow to light brown (Plate 4. 3).

**Table 4.4 Vigour Index of sorghum seeds due to treatment with selected endophytes**

| Sl. No. | Isolate | *Per cent Germination | *Shoot length (cm) | *Root length (cm) | Vigour Index (VI) | Score |
|---|---|---|---|---|---|---|
| **1** | **EB-8** | **93.33** | **4.81** | **2.58** | **6.89** | **4** |
| **2** | **EB-12** | **86.67** | **3.17** | **2.98** | **5.33** | **4** |
| **3** | **EB-13** | **86.67** | **2.87** | **2.18** | **4.37** | **4** |
| **4** | **EB-14** | **90.00** | **3.50** | **2.22** | **5.14** | **4** |
| **5** | **EB-15** | **96.67** | **4.97** | **2.68** | **7.39** | **4** |
| **6** | **EB-26** | **86.67** | **4.13** | **2.79** | **5.99** | **4** |
| **7** | **EB-31** | **93.33** | **5.12** | **2.12** | **6.75** | **4** |
| **8** | **EB-32** | **86.67** | **2.28** | **1.91** | **3.63** | **3** |
| **9** | ***P.f* (KAU)** | **90.00** | **3.91** | **3.21** | **6.40** | **4** |
| **10** | **Control** | **83.33** | **2.32** | **1.41** | **3.10** | **3** |

* Mean of three replications, *P.f- Pseudomonas fluorescens* (KAU)

Score: VI of >1<2 = 1; VI of >2<3 = 2; VI of >3<4 = 3; VI of >4 = 4

**Plate 4.3 HCN Production by endophytic isolate EB-31**

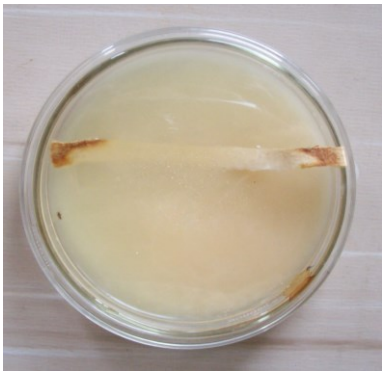
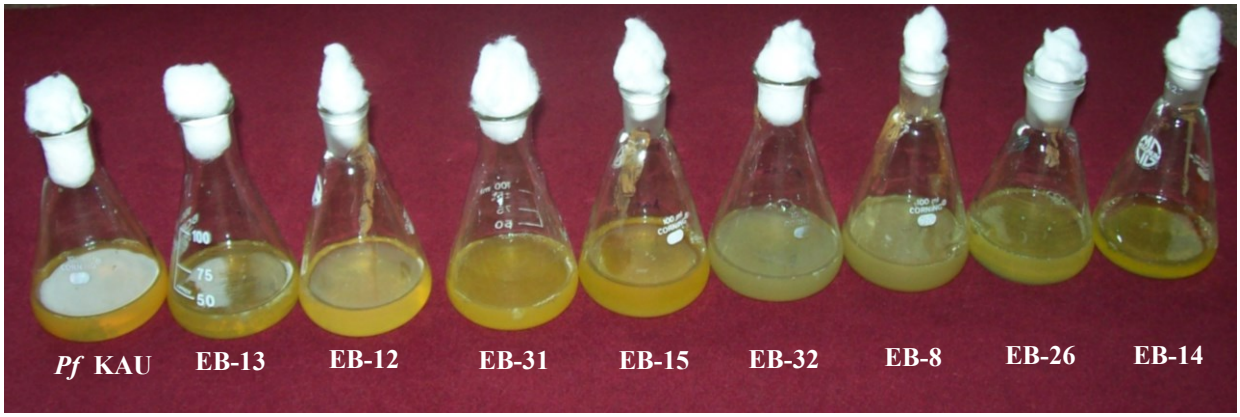


**Plate 4.4 Production of ammonia by the endophytic isolates**



### 4.8.3 Production of Ammonia

Production of ammonia by endophytes was detected by change in colour of Peptone broth media from yellow to brown on addition of Nessler's reagent. Based on colour intensity the isolates were scored. Different isolates produced varying levels of ammonia (Table 4.5 & Plate 4.4). EB-31 and EB-15 showed highest production of ammonia and were scored 4. *P. fluorescens* (KAU), EB-12, EB-13, EB-14 and EB-26 showed moderate amount of production and scored as 3. The least production of ammonia as evidenced by a faint yellow colour was noticed with EB-8 and EB-32 and was scored as 2.

### 4.8.4 Phosphorous (P) solubilization

The P solubilization ability of endophytes was tested in Pikovaskya's medium along with reference culture of *P. fluorescens (*KAU). Only four isolates *viz.*, EB-5, EB-31, EB-12, EB-26 and *P. fluorescens* (KAU) showed P solubilization ability on Pikovaskya's medium. From Table 4.6, it is clear that, EB-15 showed the maximum diameter of clearing zone , indicating its efficient P solubilizing ability (10 mm) followed by EB-31 and EB-12 (Plate 4.5).

### 4.8.5 Production of Indole Acetic Acid

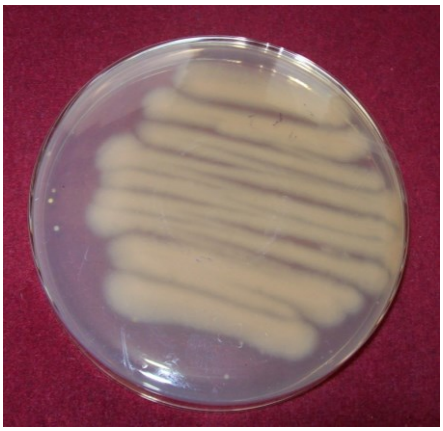
A quantitative test was conducted to know the ability of selected endophytic bacteria in producing Indole Acetic acid (IAA). From data furnished in the Table 4.7, it is clear that, all the isolates produced varying levels of IAA ranging from 7.02 – 200.00 µg $ml^{-1}$(Fig.4.2). The maximum amount of IAA was produced by EB-15 (200.0µg $ml^{-1}$) and scored as 4. It is followed by EB-26 (190.00µg $ml^{-1}$) and EB-31 (180.33µg $ml^{-1}$) also ranked as 4. Moderately high amount of IAA was produced by EB-32 (75.00µg $ml^{-1}$) and EB-12 (55.67µg $ml^{-1}$) and therefore ranked 2. However other isolates EB-13 (7.02µg $ml^{-1}$) EB-14 (15.00µg $ml^{-1}$) and EB-8 (30.00µg $ml^{-1}$) produced relatively low amount of IAA.

**Plate 4.5 Zone of Phosphorous solubilization produced by EB-15 and EB-31**

**EB-15**

**EB-31**

**Plate 4.6 Siderophore production by EB-15 and EB- 31**



**EB-31**

**EB-15**

**Table 4.6. Phosphorous solubilization by selected endophytes of anthurium**

| SI.No. | Isolate | *P solubilization zone (mm) | Score |
|---|---|---|---|
| **1** | **EB 8** | **0** | **0** |
| **2** | **EB 12** | **7.5** | **3** |
| **3** | **EB 13** | **0** | **0** |
| **4** | **EB 14** | **0** | **0** |
| **5** | **EB 15** | **10.0** | **4** |
| **6** | **EB 26** | **6.0** | **2** |
| **7** | **EB 31** | **8.3** | **3** |
| **8** | **EB 32** | **0** | **0** |
| **9** | ***P.fluorescens* (KAU)** | **7.3** | **3** |
| **10** | **Control** | **0** | **0** |

*Mean of three replications

Score: >1<3 mm = 1; >3<6 mm = 2; >6<9 mm = 3 and >9 mm = 4

**Table: 4.5 Production of ammonia by selected endophytes of anthurium**

| Sl. No. | Isolate | Score |
|---|---|---|
| **1** | **EB-8** | **2** |
| **2** | **EB-12** | **3** |
| **3** | **EB-13** | **3** |
| **4** | **EB-14** | **3** |
| **5** | **EB-15** | **4** |
| **6** | **EB-26** | **3** |
| **7** | **EB-31** | **4** |
| **8** | **EB-32** | **2** |
| **9** | ***P. fluorescens* (KAU)** | **3** |
| **10** | **Control** | **1** |

Score chart: Nil:1, Low: 2, Medium: 3, High: 4

**Table 4.7: Production of Indole Acetic Acid by selected endophytes of anthurium**

| SI. No. | Isolates | IAA (µg/ml) | Score |
|---|---|---|---|
| **1** | **EB-8** | **30.00** | **1** |
| **2** | **EB-12** | **55.67** | **2** |
| **3** | **EB-13** | **7.02** | **1** |
| **4** | **EB-14** | **15.00** | **1** |
| **5** | **EB-15** | **200.00** | **4** |
| **6** | **EB-26** | **190.33** | **4** |
| **7** | **EB-31** | **180.33** | **4** |
| **8** | **EB-32** | **75.00** | **2** |
| **9** | **Control** | **-** | **0** |

*Mean of three replications

Score : .>0<50 µg/ml =1; >50<100 µg/ml=2; >100<150 µg/ml =3; >150 µg/ml =4

### 4.9 Detection of Siderophores

The eight endophytes along with the reference culture of *P. fluorescens* (KAU) were tested for their capacity to produce siderophores using UV fluorescence method. All the endophytes except EB-12 showed fluorescence under UV light. Iron dependent production of siderophores was also estimated. The endophytes and reference culture of standard *P. fluorescens* (KAU) were grown on King's B medium supplemented with $FeCl_3.6H_2O$ at two different concentrations. It was observed that, isolates produced abundant fluorescent pigment in medium not supplemented with $FeCl_3.6H_2O$. However, the rate of production of fluorescent pigment was less in medium incorporated with $FeCl_3.6H_2O$ .(Plate 4.6)

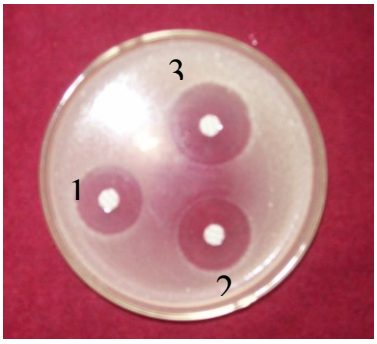
### 4.10 Compatibility of selected endophytes to plant protection chemicals and plant nutrients

The compatibility of endophytes to antibiotics, fungicides, insecticides and fertilizers were studied.

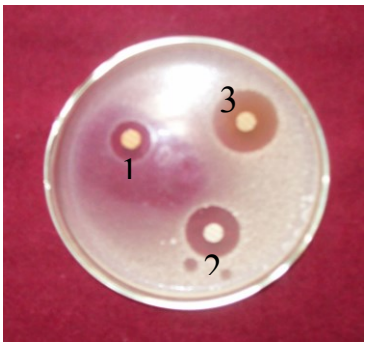
#### 4.10.1 Antibiotics

Antibiotics *viz.,* Chloramphenicol, Rifampicin at three concentrations; Ampicillin, Gentamycin, Kanamycin, Streptomycin and Tetracycline at two concentrations and Penicillin G at one concentration were used for the study. The results are presented in (Table 4.8). EB-8 differed significantly in its response to antibiotics at various concentrations. Different concentrations of all antibiotics tested recorded a per cent inhibition ranging from 8.06 - 23.46. The higher concentration of Chloramphenicol which was on par with Gentamycin recorded a per cent inhibition of 23.46. The lowest concentration of Ampicillin tested recorded a per cent inhibition of 8.06 which was on par with Tetracycline. EB-12

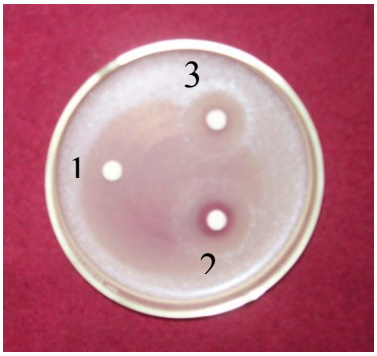
**Plate 4.7 Sensitivity of endophytes to Chloramphenicol**



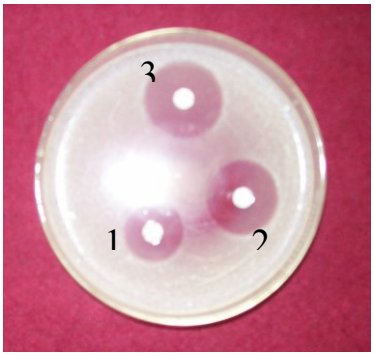

**EB-8**



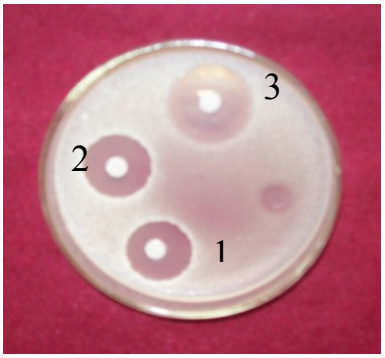

**EB-12**



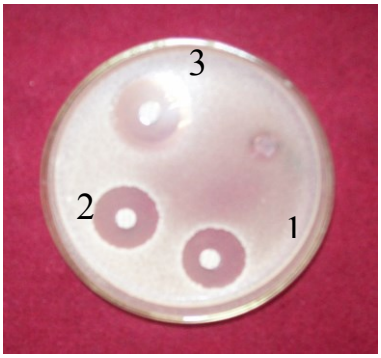

**EB-13**



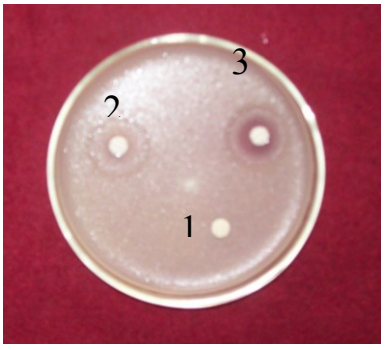

**EB-14**



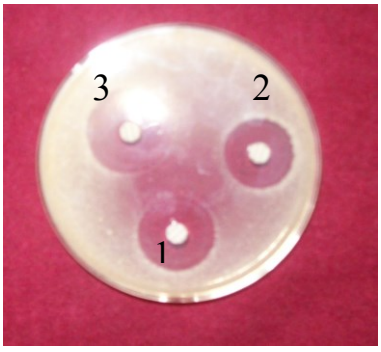

**EB-15**




**EB-26**




**EB-31**




**EB-32**

1. Chloramphenicol 0.1 ppm
2. Chloramphenicol 0.25 ppm
3. Chloramphenicol 0.50 ppm

**Table 4.8 *In vitro* compatibility of selected endophytes to antibiotics** (Contd……)

| Sl No | Antibiotics | Concentration (ppm) | EB – 8 | | EB – 12 | | EB – 13 | | EB – 15 | | EB- 26 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control |
| 1 | Chloramphenicol | 0.1 | 13.33 | 14.66$^{de}$ (3.89) | 12.33 | 13.56$^{c}$ (3.74) | 10.33 | 11.36$^{c}$ (3.44) | 13.33 | 14.66$^{e}$ (3.89) | 10.67 | 11.73$^{b}$ (3.49) |
| | | 0.25 | 16.67 | 18.33$^{g}$ (4.33) | 17.00 | 18.70$^{g}$ (4.38) | 12.00 | 13.20$^{d}$ (3.70) | 15.33 | 16.86$^{f}$ (4.16) | 12.33 | 13.56$^{c}$ (3.74) |
| | | 0.5 | 21.33 | 23.46$^{j}$ (4.89) | 20.67 | 22.73$^{h}$ (4.81) | 15.33 | 16.86$^{fg}$ (4.16) | 17.33 | 19.06$^{g}$ (4.42) | 19.33 | 21.26$^{gh}$ (4.66) |
| 2 | Gentamycin | 0.1 | 18.33 | 20.16$^{hi}$ (4.54) | 17.67 | 19.43$^{g}$ (4.46) | 16.33 | 17.96$^{gh}$ (4.29) | 14.67 | 16.13$^{f}$ (4.07) | 17.33 | 19.06$^{f}$ (4.42) |
| | | 0.3 | 21.00 | 23.10$^{j}$ (4.85) | 22.33 | 24.56$^{h}$ (5.00) | 18.33 | 20.16$^{i}$ (4.54) | 17.00 | 18.70$^{g}$ (4.38) | 19.67 | 21.63$^{h}$ (4.70) |
| 3 | Rifampicin | 0.05 | 10.33 | 11.36$^{b}$ (3.44) | 13.67 | 15.03$^{de}$ (3.94) | 10.33 | 11.36$^{c}$ (3.44) | 11.33 | 12.46$^{d}$ (3.60) | 13.33 | 14.66$^{cd}$ (3.89) |
| | | 0.15 | 14.00 | 15.40$^{e}$ (3.98) | 18.33 | 20.16$^{g}$ (4.54) | 15.00 | 16.50$^{f}$ (4.12) | 18.00 | 19.80$^{g}$ (4.50) | 15.67 | 17.23$^{e}$ (4.21) |
| | | 0.3 | 19.00 | 20.90$^{i}$ (4.62) | 21.33 | 23.46$^{hi}$ (4.89) | 17.00 | 18.70$^{h}$ (4.38) | 20.33 | 22.36$^{h}$ (4.78) | 18.00 | 19.80$^{fgh}$ (4.50) |
| 4 | Kanamycin | 0.05 | 12.00 | 13.20$^{c}$ (3.69) | 15.33 | 16.86$^{f}$ (4.16) | 10.33 | 11.36$^{c}$ (3.44) | 0.00 | 0.0$^{a}$ (0.70) | 13.00 | 14.30$^{cd}$ (3.84) |
| | | 0.3 | 17.33 | 19.06$^{gh}$ (4.42) | 17.00 | 18.70$^{g}$ (4.38) | 12.00 | 13.20$^{d}$ (3.69) | 8.00 | 9.16$^{c}$ (3.10) | 19.33 | 21.26$^{gh}$ (4.66) |
| 5 | Ampicilin | 0.1 | 7.33 | 8.06$^{a}$ (2.92) | 8.33 | 9.16$^{a}$ (3.10) | 8.00 | 8.80$^{b}$ (3.04) | 7.33 | 8.06$^{b}$ (2.92) | 10.33 | 11.36$^{b}$ (3.44) |
| | | 0.2 | 13.00 | 14.30$^{cde}$ (3.84) | 14.00 | 15.40$^{def}$ (3.98) | 12.33 | 13.56$^{de}$ (3.74) | 9.00 | 9.90$^{c}$ (3.22) | 13.00 | 14.30$^{cd}$ (3.84) |
| 6 | Streptomycin sulphate | 0.1 | 15.33 | 16.86$^{f}$ (4.16) | 15.00 | 16.50$^{ef}$ (4.12) | 13.33 | 14.66$^{e}$ (3.89) | 14.33 | 15.76$^{ef}$ (4.03) | 17.76 | 19.43$^{fg}$ (4.40) |
| | | 0.25 | 18.33 | 20.16$^{h}$ (4.54) | 22.00 | 24.20$^{hi}$ (4.96) | 18.33 | 20.16$^{i}$ (4.54) | 19.33 | 21.26$^{h}$ (4.66) | 22.33 | 24.56h (5.00) |
| 7 | Tetracycline | 0.1 | 7.33 | 8.06$^{a}$ (2.92) | 10.00 | 11.00$^{b}$ (3.39) | 7.33 | 8.06$^{b}$ (2.92) | 0.00 | 0.0$^{a}$ (0.70) | 8.33 | 9.16$^{a}$ (3.10) |
| | | 0.3 | 12.33 | 13.56$^{cd}$ (3.74) | 13.33 | 14.56$^{cd}$ (3.88) | 13.00 | 14.30$^{de}$ (3.84) | 10.33 | 11.36$^{d}$ (3.44) | 14.33 | 15.76$^{de}$ (4.03) |
| 8 | Penicillin G | 0.1 | 13.67 | 15.03$^{e}$ (3.94) | 14.33 | 15.76$^{def}$ (4.03) | 0.00 | 0.00$^{a}$ (0.70) | 0.00 | 0.0$^{a}$ (0.70) | 14.00 | 15.40$^{d}$ (3.98) |

In each column figure followed by same letter do not differ significantly according to DMRT.
Figures in parenthesis are $\sqrt{x} + 0.5$ transformed value.

**Table 4.8 *In vitro* compatibility of selected endophytes to antibiotics**

| Sl No. | Antibiotics | Concentration (ppm) | EB-26 | | EB-31 | | EB-32 | | *Pf* KAU | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control |
| 1 | Chloramphenicol | 0.1 | 10.33 | $11.36^{b}$ (3.43) | 11.67 | $12.83^{b}$ (3.64) | 11.00 | $12.10^{cd}$ (3.54) | 12.33 | $13.56^{c}$ (4.20) |
| | | 0.25 | 12.67 | $13.93^{c}$ (3.79) | 13.67 | $15.03^{de}$ (3.93) | 15.33 | $16.86^{e}$ (4.16) | 15.67 | $17.23^{f}$ (4.20) |
| | | 0.8 | 15.33 | $16.86^{de}$ (4.16) | 17.00 | $18.70^{g}$ (4.38) | 18.67 | $20.53^{fg}$ (4.58) | 18.33 | $20.16^{gh}$ (4.24) |
| 2 | Gentamycin | 0.1 | 16.33 | $17.96^{e}$ (4.29) | 17.33 | $19.06^{g}$ (4.42) | 15.67 | $17.23^{e}$ (4.20) | 17.67 | $19.43^{gh}$ (4.46) |
| | | 0.3 | 19.67 | $21.63^{f}$ (4.70) | 19.33 | $21.26^{h}$ (4.66) | 17.33 | $19.06^{f}$ (4.42) | 20.33 | $22.36^{i}$ (4.78) |
| 3 | Rifampicin | 0.05 | 12.33 | $13.56^{c}$ (3.74) | 11.33 | $12.46^{b}$ (3.60) | 11.33 | $12.46^{d}$ (3.60) | 7.33 | $8.06^{b}$ (2.92) |
| | | 0.15 | 16.67 | $18.33^{e}$ (4.33) | 13.33 | $14.00^{cd}$ (3.89) | 15.00 | $16.50^{e}$ (4.12) | 12.33 | $13.56^{e}$ (3.74) |
| | | 0.3 | 17.00 | $18.70^{e}$ (4.38) | 18.00 | $19.80^{gh}$ (4.50) | 18.33 | $20.16f^{g}$ (4.54) | 17.00 | $18.70^{fg}$ (4.38) |
| 4 | Kanamycin | 0.05 | 12.00 | $9.90^{ab}$ (3.22) | 15.33 | $13.20^{bc}$ (3.69) | 10.33 | $9.90^{b}$ (3.22) | 9.33 | $10.26^{c}$ (3.28) |
| | | 0.3 | 17.33 | $15.76^{d}$ (4.03) | 17.00 | $18.70^{g}$ (4.38) | 12.00 | 16.86e (4.16) | 8.33 | $12.10^{d}$ (3.54) |
| 5 | Ampicilin | 0.1 | 10.00 | $11.00^{b}$ (3.38) | 11.00 | $12.10^{b}$ (3.54) | 10.00 | $11.00^{bc}$ (3.38) | 8.33 | $9.16^{bc}$ (3.10) |
| | | 0.25 | 12.33 | $13.56^{c}$ (3.74) | 13.33 | $14.66^{cd}$ (3.89) | 12.33 | $13.56^{d}$ (3.74) | 12.00 | $13.20^{de}$ (3.69) |
| 6 | Streptomycin sulphate | 0.1 | 15.33 | $16.86^{de}$ (4.16) | 15.33 | $16.86^{f}$ (4.16) | 14.67 | $16.13^{e}$ (4.07) | 13.33 | $14.67^{e}$ (3.89) |
| | | 0.25 | 21.00 | $23.10^{f}$ (4.85) | 19.67 | $21.63^{h}$ (4.70) | 19.00 | $20.90^{g}$ (4.62) | 19.00 | $20.90^{hi}$ (4062) |
| 7 | Tetracycline | 0.1 | 8.00 | $8.80^{a}$ (3.04) | 9.00 | $9.90^{a}$ (3.22) | 0.00 | $0.00^{a}$ (0.70) | 0.00 | $0.00^{a}$ (0.70) |
| | | 0.3 | 12.33 | $13.56^{c}$ (3.74) | 15.00 | $16.50^{ef}$ (4.12) | 12.00 | $13.20^{d}$ (03.69) | 0.00 | $0.00^{a}$ (0.70) |
| 8 | Penicillin G | 0.1 | 12.33 | $13.56^{c}$ (3.74) | 14.00 | $15.40^{def}$ (3.98) | 0.00 | $0.00^{a}$ (0.70) | 0.00 | $0.00^{a}$ (0.70) |

In each column figure followed by same letter do not differ significantly according to DMRT.

Figures in parenthesis are $\sqrt{x} + 0.5$ transformed value.

*Pf*(KAU) : *Pseudomonas fluorescens* (KAU)

(Contd.....)

differed significantly in its response to antibiotics at various concentrations. Different concentrations of all antibiotics tested recorded a per cent inhibition ranging from 9.16-24.56. The maximum per cent inhibition was recorded with higher concentration of Gentamycin (24.56) and least by Ampicillin (9.16). In case of EB-13, higher concentration of Gentamycin which was on par with Streptocycline recorded a maximum per cent inhibition of 20.16. It was found compatible with Penicillin G and recorded a per cent inhibition ranging from 8.06-20.16 for all other antibiotics tested. EB-14 was found compatible with Penicillin G and lowest concentration of Tetracycline and Kanamycin. Other antibiotics at different dosage caused an inhibition ranging from 0-22.36 per cent. EB-15 differed significantly in its response to antibiotics at various concentrations. Different concentrations of all antibiotics tested recorded a per cent inhibition ranging from 9.16-24.56. Highest inhibition was recorded with higher concentration of Streptomycin sulphate and least with lower concentration of Tetracycline. In case of EB-26, highest inhibition was recorded with higher concentration of Streptomycin sulphate which was on par with Gentamycin and recorded a per cent inhibition of 23.10. Least inhibition was recorded with lower concentration of Tetracycline (8.80). EB-31 also differed significantly in its response to antibiotics at various concentrations. Highest inhibition was recorded higher concentration of Streptomycin sulphate (19.67) and least with lower concentration of Tetracycline (9). EB-32 was found compatible with Pencillin G and lowest concentration of Tetracycline. Maximum inhibition was recorded with higher concentration of Streptomycin sulphate (20.90) and least with lowest concentration of Kanamycin (9.90). *P. fluorescens* (KAU) was compatible with Pencillin G and Tetracycline. Maximum inhibition was recorded with Gentamycin (22.36) and least by lowest concentration of Rifampicin (8.06).

The *in vitro* compatibility of endophytes to Streptocycline and turmeric-sodium bicarbonate mixture was also studied and the results are presented in the (Table 4.9). All isolates, except EB-12 and EB-15 were found compatible with lower concentration of turmeric-sodium bicarbonate mixture. EB-13, EB-26,

**Table 4.9 *In vitro* compatibility of selected endophytes with Streptocycline and Turmeric-sodium bicarbonate mixture**

| Chemical | Concentration (ppm) | EB - 8 | | EB - 12 | | EB - 13 | | EB – 14 | | EB – 15 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control |
| Streptocycline | 200 | 13.33 | 14.67$^{c}$ (3.89) | 12.33 | 13.56$^{b}$ (3.74) | 10.36 | 11.36$^{b}$ (3.44) | 11.67 | 12.83$^{c}$ (3.65) | 10.67 | 11.73$^{a}$ (3.49) |
| | 300 | 15.67 | 17.23$^{d}$ (4.21) | 17.00 | 18.70$^{d}$ (4.38) | 11.67 | 12.83$^{c}$ (3.65) | 15.33 | 16.86$^{d}$ (4.16) | 12.33 | 13.56$^{b}$ (3.74) |
| | 400 | 21.33 | 23.46$^{e}$ (4.89) | 19.67 | 21.63$^{e}$ (4.70) | 14.67 | 16.13$^{d}$ (4.07) | 17.33 | 19.06$^{e}$ (4.42) | 19.33 | 21.26$^{c}$ (4.66) |
| Turmeric-sodium bicarbonate mixture | 500 | 0.00 | 0.00$^{a}$ (0.70) | 11.33 | 12.46$^{a}$ (3.60) | 0.00 | 0.00$^{a}$ (0.70) | 0.00 | 0.00$^{a}$ (0.70) | 10.67 | 11.73$^{a}$ (3.49) |
| | 1000 | 11.67 | 12.86$^{b}$ (3.65) | 13.33 | 14.66$^{c}$ (3.89) | 10.67 | 11.73$^{b}$ (3.49) | 10.33 | 11.36$^{b}$ (3.44) | 10.67 | 12.83$^{ab}$ (3.65) |
| | 1500 | 12.67 | 13.93$^{b}$ (3.79) | 13.67 | 15.03$^{c}$ (3.94) | 12.33 | 13.56$^{c}$ (3.74) | 12.67 | 11.73$^{b}$ (3.49) | 12.67 | 13.93$^{b}$ (3.79) |

In each column figure followed by same letter do not differ significantly according to DMRT.
Figures in parenthesis are $\sqrt{x} + 0.5$ transformed value

**Table 4.9 *In vitro* compatibility of selected endophytes with Streptocycline and Turmeric sodium bicarbonate mixture**

| Chemicals | Concentration (ppm) | EB-26 | | EB-31 | | EB-32 | | *Pf* (KAU) | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control |
| Streptocycline | 200 | 10.33 | 11.36[b] (3.44) | 11.33 | 12.46[b] (3.60) | 11.00 | 12.83[c] (3.65) | 11.67 | 12.83[c] (3.65) |
| | 300 | 11.67 | 12.83[c] (3.65) | 12.67 | 13.93[c] (3.79) | 14.67 | 16.13[d] (4.07) | 14.67 | 16.13[d] (4.07) |
| | 400 | 15.33 | 16.86[d] (4.16) | 17.00 | 18.70[e] (4.38) | 17.67 | 20.16[e] (4.54) | 18.33 | 20.16[e] (4.54) |
| Turmeric sodium bicarbonate mixture | 500 | 0.00 | 0.00[a] (0.70) | 0.00 | 0.00[a] (0.70) | 0.00 | 0.00[a] (0.70) | 0.00 | 0.00[a] (0.70) |
| | 1000 | 10.00 | 11.00[b] (3.39) | 11.67 | 12.83[b] (3.65) | 0.00 | 0.00[a] (0.70) | 0.00 | 0.00[a] (0.70) |
| | 1500 | 12.33 | 13.56[c] (3.74) | 12.33 | 15.40[d] (3.98) | 12.67 | 13.93[b] (3.79) | 10.33 | 11.36[b] (3.44) |

In each column, figures followed by same letter do not differ significantly according to DMRT.

Figure in parenthesis are $\sqrt{x} + 0.5$ transformed values.

*Pf* (KAU): *Pseudomonas fluorescens* (KAU)

EB-31 and EB-32 were found sensitive to all the three concentrations of Streptocycline and slightly sensitive to higher two concentrations of turmeric-sodium bicarbonate mixture. EB-8 and EB-14 were highly sensitive to all the three concentrations of Streptocycline compared to other isolates and least sensitive to higher concentrations of turmeric- sodium bicarbonate mixture. *P. fluorescens* (KAU) was found compatible with lower two concentrations of turmeric- sodium bicarbonate mixture and slightly sensitive to the highest concentration. It was sensitive to all the three concentrations of Streptocycline.In general, among all the three concentrations of Streptocycline tested, highest concentration recorded greater inhibition. All the isolates were found to be slightly sensitive to turmeric sodium bicarbonate mixture.

### 4.10.2 Fungicides

Six fungicides *viz.,* Bavistin, Indofil M 45, Saaf, Contaf and Akomin 40, each at three different concentrations, were evaluated for their compatibility with selected endophytes and data are presented.(Table 4.10) (Plate 4.8). All the isolates were found to be compatible with Bavistin, Akomin and Contaf at all the three concentrations tested. But they were highly sensitive to higher concentrations of Mancozeb which was on par with Saaf. All isolates were moderately sensitive to Captaf.

### 4.10.3 Insecticides

Insecticides *viz.,* Malathion, Rogor, Ekalux, Hostathion, Monocrotophos and Classic 20 at three different concentrations were used for the *in vitro* evaluation and it was observed that none of them had inhibitory effect against any of the isolates.

**Table 4.8 *In vitro* compatibility of selected endophytes to antibiotics**

| Sl No. | Antibiotics | Concentration (ppm) | EB-26 | | EB-31 | | EB-32 | | *Pf* KAU | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control |
| 1 | Chloramphenicol | 0.1 | 10.33 | $11.36^{b}$ (3.43) | 11.67 | $12.83^{b}$ (3.64) | 11.00 | $12.10^{cd}$ (3.54) | 12.33 | $13.56^{c}$ (4.20) |
| | | 0.25 | 12.67 | $13.93^{c}$ (3.79) | 13.67 | $15.03^{de}$ (3.93) | 15.33 | $16.86^{e}$ (4.16) | 15.67 | $17.23^{f}$ (4.20) |
| | | 0.8 | 15.33 | $16.86^{de}$ (4.16) | 17.00 | $18.70^{g}$ (4.38) | 18.67 | $20.53^{fg}$ (4.58) | 18.33 | $20.16^{gh}$ (4.24) |
| 2 | Gentamycin | 0.1 | 16.33 | $17.96^{e}$ (4.29) | 17.33 | $19.06^{g}$ (4.42) | 15.67 | $17.23^{e}$ (4.20) | 17.67 | $19.43^{gh}$ (4.46) |
| | | 0.3 | 19.67 | $21.63^{f}$ (4.70) | 19.33 | $21.26^{h}$ (4.66) | 17.33 | $19.06^{f}$ (4.42) | 20.33 | $22.36^{i}$ (4.78) |
| 3 | Rifampicin | 0.05 | 12.33 | $13.56^{c}$ (3.74) | 11.33 | $12.46^{b}$ (3.60) | 11.33 | $12.46^{d}$ (3.60) | 7.33 | $8.06^{b}$ (2.92) |
| | | 0.15 | 16.67 | $18.33^{e}$ (4.33) | 13.33 | $14.00^{cd}$ (3.89) | 15.00 | $16.50^{e}$ (4.12) | 12.33 | $13.56^{e}$ (3.74) |
| | | 0.3 | 17.00 | $18.70^{e}$ (4.38) | 18.00 | $19.80^{gh}$ (4.50) | 18.33 | $20.16f^{g}$ (4.54) | 17.00 | $18.70^{fg}$ (4.38) |
| 4 | Kanamycin | 0.05 | 12.00 | $9.90^{ab}$ (3.22) | 15.33 | $13.20^{bc}$ (3.69) | 10.33 | $9.90^{b}$ (3.22) | 9.33 | $10.26^{c}$ (3.28) |
| | | 0.3 | 17.33 | $15.76^{d}$ (4.03) | 17.00 | $18.70^{g}$ (4.38) | 12.00 | 16.86e (4.16) | 8.33 | $12.10^{d}$ (3.54) |
| 5 | Ampicilin | 0.1 | 10.00 | $11.00^{b}$ (3.38) | 11.00 | $12.10^{b}$ (3.54) | 10.00 | $11.00^{bc}$ (3.38) | 8.33 | $9.16^{bc}$ (3.10) |
| | | 0.25 | 12.33 | $13.56^{c}$ (3.74) | 13.33 | $14.66^{cd}$ (3.89) | 12.33 | $13.56^{d}$ (3.74) | 12.00 | $13.20^{de}$ (3.69) |
| 6 | Streptomycin sulphate | 0.1 | 15.33 | $16.86^{de}$ (4.16) | 15.33 | $16.86^{f}$ (4.16) | 14.67 | $16.13^{e}$ (4.07) | 13.33 | $14.67^{e}$ (3.89) |
| | | 0.25 | 21.00 | $23.10^{f}$ (4.85) | 19.67 | $21.63^{h}$ (4.70) | 19.00 | $20.90^{g}$ (4.62) | 19.00 | $20.90^{hi}$ (4062) |
| 7 | Tetracycline | 0.1 | 8.00 | $8.80^{a}$ (3.04) | 9.00 | $9.90^{a}$ (3.22) | 0.00 | $0.00^{a}$ (0.70) | 0.00 | $0.00^{a}$ (0.70) |
| | | 0.3 | 12.33 | $13.56^{c}$ (3.74) | 15.00 | $16.50^{ef}$ (4.12) | 12.00 | $13.20^{d}$ (03.69) | 0.00 | $0.00^{a}$ (0.70) |
| 8 | Penicillin G | 0.1 | 12.33 | $13.56^{c}$ (3.74) | 14.00 | $15.40^{def}$ (3.98) | 0.00 | $0.00^{a}$ (0.70) | 0.00 | $0.00^{a}$ (0.70) |

In each column figure followed by same letter do not differ significantly according to DMRT.

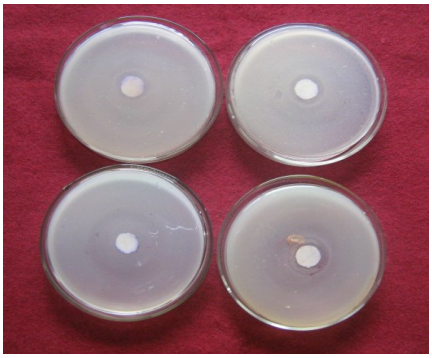
Figures in parenthesis are $\sqrt{x} + 0.5$ transformed value.

*Pf*(KAU) : *Pseudomonas fluorescens* (KAU)

(Contd.....)

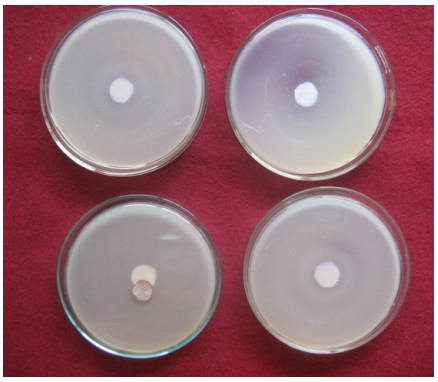
**Plate 4.7 Sensitivity of endophytes to Mancozeb**

**EB-8**



**EB-12**

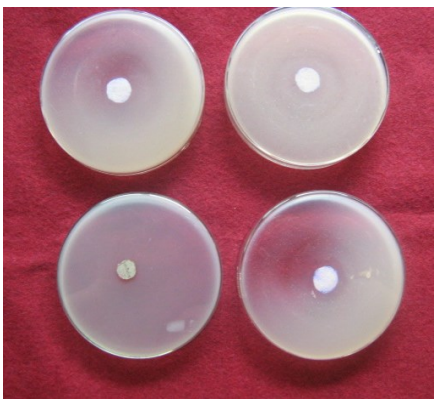
**EB-13**



**EB-14**

**EB-15**

**EB-26**



**EB-31**

**EB-32**

1. Control 2. Mancozeb 0.2% 3. Mancozeb 0.3% 4. Mancozeb 0.4%

**Table 4.10 *In vitro* compatibility of selected endophytes with fungicides**

| Sl. No. | Fungicide | Concentration (%) | EB 8 | | EB 12 | | EB 13 | | EB 14 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control |
| 1 | Mancozeb | 0.2 | 16.33 | 17.96$^{b}$ | 17.33 | 19.06$^{c}$ | 16.33 | 17.96$^{cd}$ | 16.33 | 17.96$^{de}$ |
| | | 0.3 | 18.33 | 20.17$^{c}$ | 19.00 | 20.90$^{d}$ | 18.00 | 19.80$^{e}$ | 17.00 | 18.70$^{ef}$ |
| | | 0.4 | 21.10 | 23.10$^{d}$ | 22.00 | 24.20$^{e}$ | 19.33 | 21.26$^{f}$ | 18.33 | 20.16$^{g}$ |
| 2 | Captaf | 0.5 | 13.33 | 14.67$^{a}$ | 13.33 | 14.67$^{a}$ | 11.00 | 12.10$^{a}$ | 8.33 | 9.16$^{a}$ |
| | | 0.1 | 14.33 | 15.77$^{a}$ | 15.33 | 16.86$^{b}$ | 14.00 | 15.40$^{b}$ | 11.67 | 12.83$^{b}$ |
| | | 0.2 | 16.33 | 17.60$^{b}$ | 16.67 | 18.33$^{c}$ | 15.33 | 16.86$^{c}$ | 14.33 | 15.76$^{c}$ |
| 3 | Saaf | 0.2 | 16.33 | 17.60$^{b}$ | 16.67 | 18.33$^{c}$ | 16.33 | 17.96$^{cd}$ | 16.33 | 17.96$^{de}$ |
| | | 0.3 | 16.67 | 18.33$^{b}$ | 17.67 | 19.43$^{c}$ | 16.67 | 18.33$^{d}$ | 15.67 | 17.23$^{d}$ |
| | | 0.4 | 20.33 | 22.37$^{d}$ | 21.33 | 23.46$^{e}$ | 19.33 | 21.26$^{f}$ | 17.67 | 19.43$^{fg}$ |

In each column figure followed by same letter do not differ significantly according to DMRT

**Table 4.10 *In vitro* compatibility of selected endophytes with fungicides** (Contd…..)

| Sl No. | Fungicide | Concentration (%) | EB-15 | | EB-26 | | EB-31 | | EB-32 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control | Mean diameter of inhibition zone (mm) | Pe cent inhibition over control | Mean diameter of inhibition zone (mm) | Per cent inhibition over control |
| 1 | Mancozeb | 0.2 | 17.33 | 19.06$^{d}$ | 16.33 | 17.96$^{d}$ | 17.33 | 19.06$^{c}$ | 17.33 | 19.06$^{cd}$ |
| | | 0.3 | 19.33 | 21.26$^{e}$ | 18.00 | 19.80$^{e}$ | 19.00 | 20.90$^{d}$ | 19.00 | 20.90$^{e}$ |
| | | 0.4 | 21.00 | 23.10$^{f}$ | 20.33 | 22.36$^{f}$ | 21.33 | 23.46$^{e}$ | 21.00 | 23.10$^{f}$ |
| 2 | Captaf | 0.5 | 12.33 | 13.56$^{a}$ | 12.00 | 13.20$^{a}$ | 11.33 | 12.46$^{a}$ | 12.33 | 13.56$^{a}$ |
| | | 0.1 | 15.33 | 16.86$^{b}$ | 13.67 | 15.03$^{b}$ | 14.33 | 15.76$^{b}$ | 14.67 | 16.13$^{b}$ |
| | | 0.2 | 16.33 | 17.96$^{c}$ | 14.67 | 16.13$^{c}$ | 14.67 | 16.13$^{b}$ | 16.33 | 17.96$^{c}$ |
| 3 | Saaf | 0.2 | 16.67 | 18.33$^{cd}$ | 16.67 | 18.33$^{d}$ | 17.33 | 19.06$^{c}$ | 16.67 | 18.33$^{cd}$ |
| | | 0.3 | 19.33 | 21.26$^{e}$ | 18.00 | 19.80$^{e}$ | 19.33 | 21.26$^{d}$ | 17.67 | 19.43$^{d}$ |
| | | 0.4 | 20.67 | 22.73$^{f}$ | 20.33 | 22.36$^{f}$ | 20.67 | 22.73$^{e}$ | 21.00 | 23.10$^{f}$ |

In each column figure followed by same letter do not differ significantly according to DMRT

### 4.10.4 Fertilizers

Fertilizers *viz.,* Urea, Rajphos, MOP and Factomphos were evaluated for their inhibitory effect on the endophytes. It was noticed that, none of them were inhibitory to the isolates.

## 4.11 *In vitro* sensitivity of pathogen to plant protection chemicals

A similar method as mentioned in section 4.10 was followed for testing the effect of antibiotics, fungicides and turmeric sodium bicarbonate mixture on growth of the pathogen. Streptocycline, Captaf, Mancozeb and Turmeric sodium bicarbonate mixture were used for *in vitro* testing and results are presented in (Table 4.11). The plant protection chemicals differed significantly in their sensitivity towards the pathogen. The maximum sensitivity was recorded with 400 ppm streptocycline followed by 4000 ppm of Mancozeb. Turmeric sodium bicarbonate mixture and Captan showed comparitively less inhibitory effect.

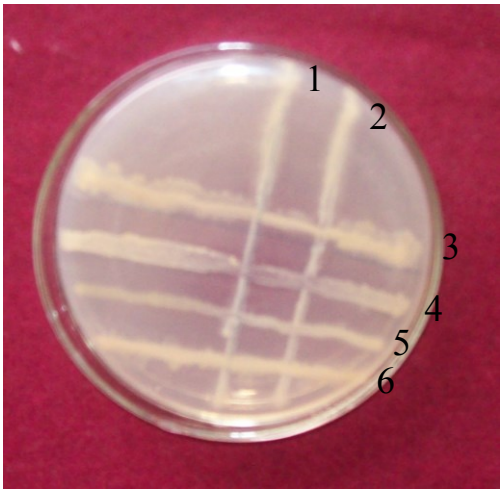
## 4.12 Mutual compatibility of selected endophytes

To assess the mutual compatibility of the endophytes, the organisms were streaked perpendicular to each other as described in section 3. It was observed that all the isolates were compatible with each other as none of the isolate showed a lysis at the juncture between the antagonists (Plate 4.9).

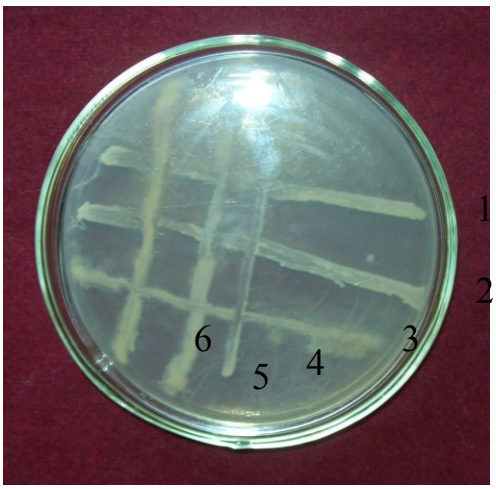
## 4.13. Pot culture evaluation of potential endophytes against the disease

A pot culture experiment was conducted to evaluate the efficiency of selected endophytes and standard culture of *P. fluorescens* (KAU) for the management of bacterial blight disease in comparison with standard recommendations like use of Turmeric sodium bicarbonate mixture and antibiotic Streptocycline (Plate 4.10)

**Plate 4.9 Mutual compatibilitiy of endophytes**




1.EB-15 2.EB-31 3. EB- 8
4.EB-12 5.EB-13 6.EB-26




1.EB-14 2.EB-13 3.EB-32
4.*Pf* KAU 5. EB-15 6.EB-31

**Table 4.11 *In vitro* inhibitory effect of plant protection chemicals against the pathogen**

| Sl. No. | Treatments | Concentration (ppm) | Mean diameter of inhibition zone (mm) | Per cent inhibition |
|---|---|---|---|---|
| 1 | Streptocycline | 200 | 18.67 | 20.53[d] |
| | | 300 | 19.33 | 22.73[e] |
| | | 400 | 21.33 | 32.26[g] |
| 2 | Mancozeb | 2000 | 17.33 | 19.06[c] |
| | | 3000 | 17.67 | 19.43[c] |
| | | 4000 | 20.67 | 24.56[f] |
| 3 | Captan | 500 | 12.33 | 13.93[a] |
| | | 1000 | 14.67 | 16.13[b] |
| | | 1500 | 19.33 | 21.26[c] |
| 4 | Turmeric-sodium bicarbonate mixture | 500 | 12.67 | 14.30[a] |
| | | 1000 | 14.37 | 16.86[b] |
| | | 1500 | 17.67 | 19.43[c] |

In each column figure followed by same letter do not differ significantly according to DMRT

**Pot culture experiment**

$T_1$

$T_2$

$T_3$

$T_4$

$T_5$

$T_6$

$T_7$

$T_8$

$T_9$

**4.9.** T1 –EB 12

T2 –EB -13

All T3 –EB-15 mixture(10:1)

afte

inoc T4 –EB- 26

con

T6 – Pf (KAU)

T7 – Streptocycline(300ppm)

T8- Turmeric sodium bicarbonate

T9 - Control

**Effect of Pre inoculation spraying on diseases severity**

Two pre-inoculation spraying were given at five days intervals with all tratments. All the plants which were given pre-inoculation sprays developed diseases symptom only after artificial inoculation of the pathogen which was done five days second pre inoculation spray. Diseases incidence and severity was less in treated plants compared to control

**4.10. Per cent disease incidence**

It was observed that there was significant difference among the treatments on the per cent disease incidence after five days of inoculation (Table 4.12), (Fig. 4.3). The lowest incidence was observed in plants sprayed with endophyte EB-15 ($T_3$ - 61.00 per cent) followed by $T_7$ (67.00 per cent) and $T_5$ (72 per cent) where as cent per cent incidence was recorded for control on the same day. All other treatments showed per cent disease incidence of 90.00 ($T_1$, $T_2$, $T_4$ and $T_6$) or above (95 per cent in $T_8$). More or less similar trend was observed after ten days of inoculation also, with $T_3$ showing the least disease incidence (80 per cent). It was followed by $T_7$ and $T_5$ both recording 95 per cent disease incidence. All the plants in different treatments took cent per cent disease incidence by fifteenth day of inoculation with the pathogen.

**4.11. Per cent disease severity**

Data on the per cent severity of disease revealed that there was significant difference among the treatments (Table 4.13 & Fig.4.4). The treatment $T_3$ (EB-15) was the most effective with least PDS during the study (*ie*., from five days after inoculation to 30 days after inoculation) and that was on par with $T_1$, $T_5$ and $T_7$ . At five days after inoculation $T_3$ (EB-15) was followed by $T_7$ (Streptocycline), $T_5$ (EB-31) and $T_1$ (EB-12) respectively. However they were on par with each

**Fig.4.3: Effect of various treatments on the incidence of bacterial blight of anthurium**

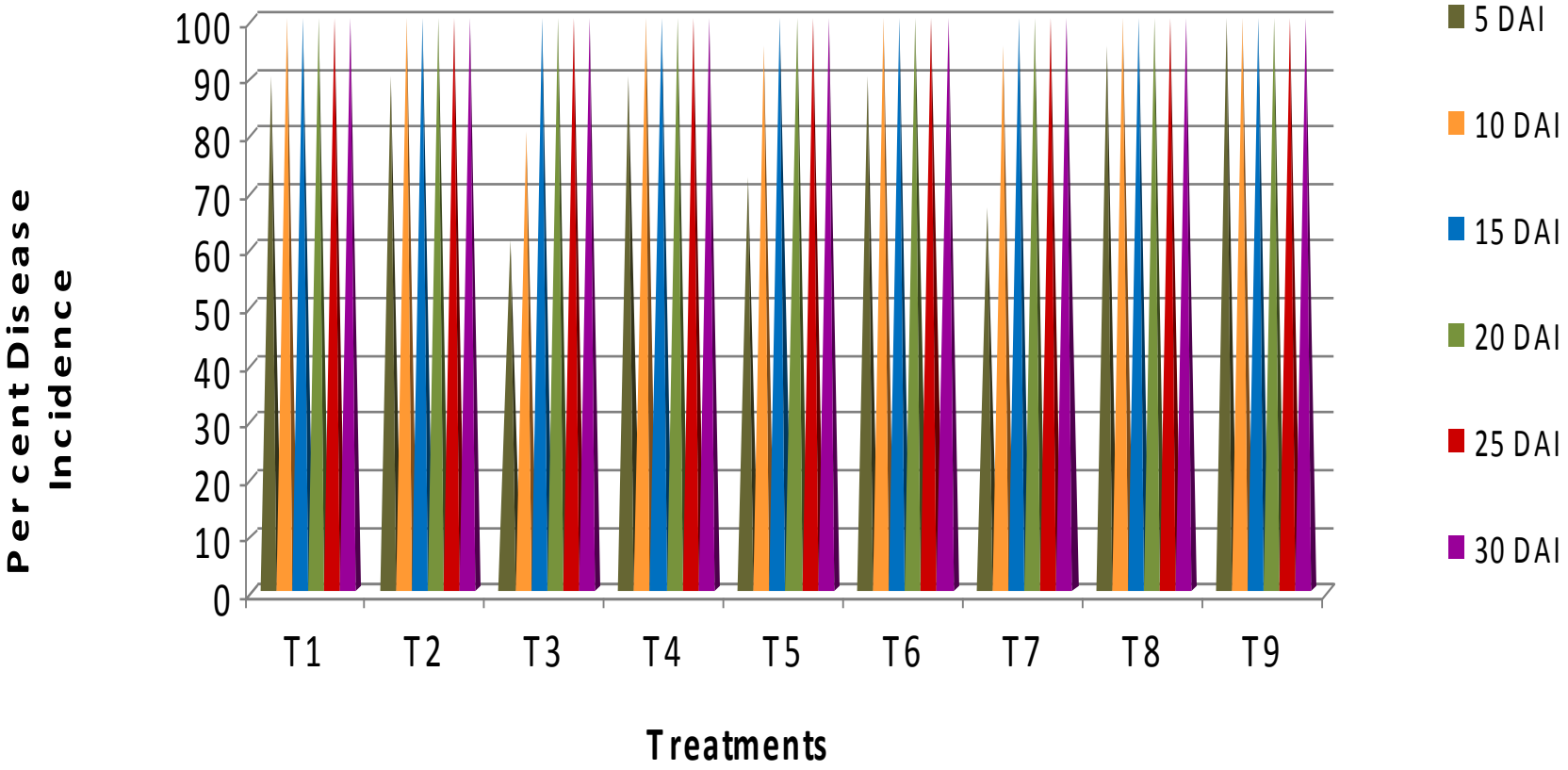


**Table 4.12 Effect of various treatments on the incidence of bacterial blight of anthurium**

| Treatments | Treatment details | Per cent disease incidence* | | | | | |
|---|---|---|---|---|---|---|---|
| | | 5 DAI | 10 DAI | 15 DAI | 20 DAI | 25 DAI | 30 DAI |
| $T_1$ | EB-12 | 90 (9.49)$^{ab}$ | 100 (10.02)$^{a}$ | 100 | 100 | 100 | 100 |
| $T_2$ | EB-13 | 90 (9.49)$^{ab}$ | 100 (10.02)$^{a}$ | 100 | 100 | 100 | 100 |
| $T_3$ | EB-15 | 61 (7.69)$^{c}$ | 80 (8.95)$^{b}$ | 100 | 100 | 100 | 100 |
| $T_4$ | EB-26 | 90 (9.49)$^{ab}$ | 100 (10.02)$^{a}$ | 100 | 100 | 100 | 100 |
| $T_5$ | EB-31 | 72 (8.45)$^{bc}$ | 95 (9.75)$^{a}$ | 100 | 100 | 100 | 100 |
| $T_6$ | *Pf* (KAU) | 90 (9.49)$^{ab}$ | 100 (10.02)$^{a}$ | 100 | 100 | 100 | 100 |
| $T_7$ | Streptocycline (300 ppm) | 67 (8.19)$^{c}$ | 95 (9.75)$^{a}$ | 100 | 100 | 100 | 100 |
| $T_8$ | Turmeric + sodium bicarbonate mixture (10:1) | 95 (9.76)$^{a}$ | 100 (10.02)$^{a}$ | 100 | 100 | 100 | 100 |
| $T_9$ | Control | 100 (10.02)$^{a}$ | 100 (10.02)$^{a}$ | 100 | 100 | 100 | 100 |

*Mean of three replications
DAI: Days after inoculation
Figures in parenthesis are square root transformed values.
In each column, figures followed by same letters do not differ significantly according to DMRT

**Table: 4.13 Effect of endophytes on the severity of bacterial blight of anthurium**

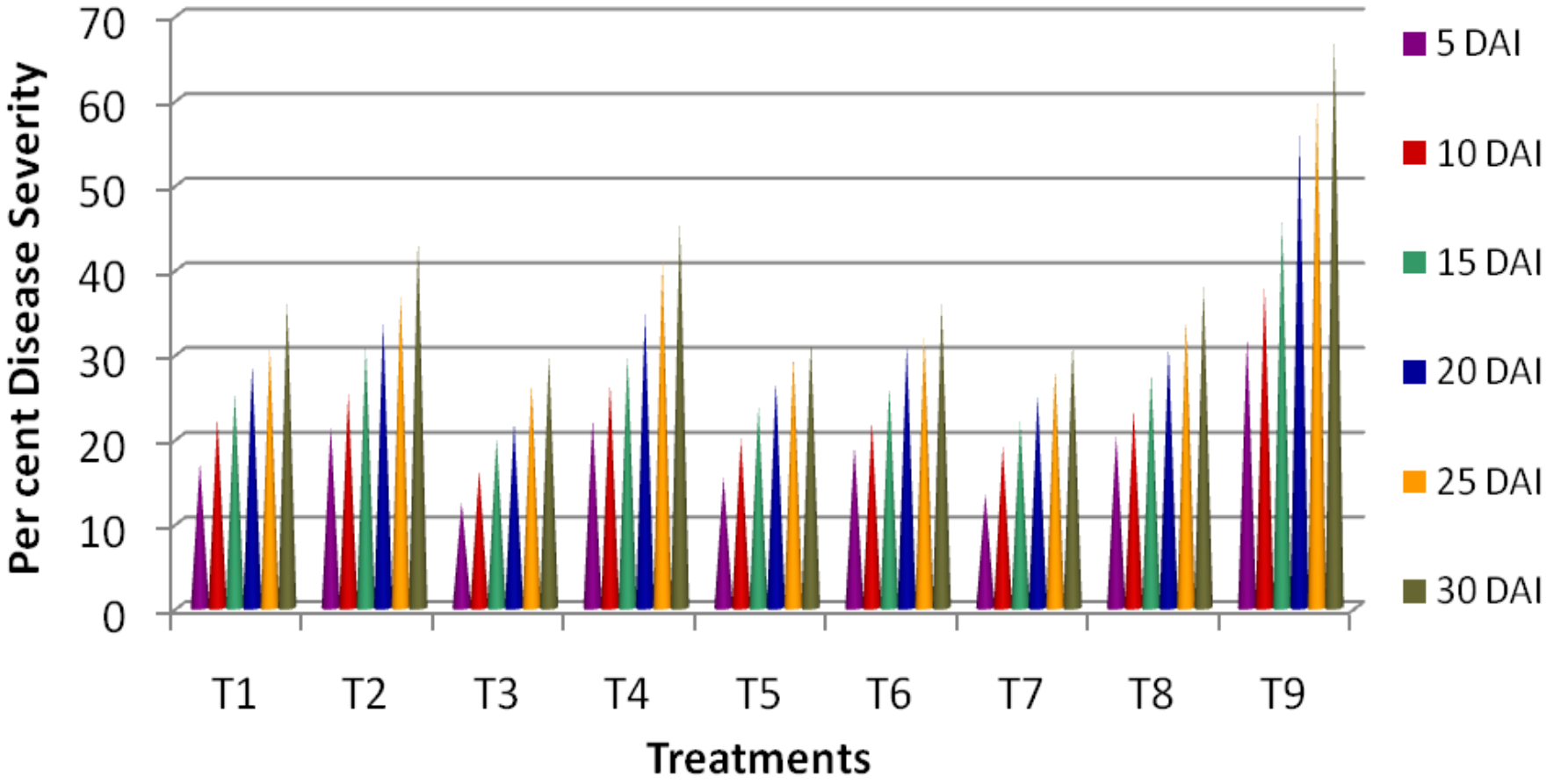
| Treat-ments | Treatment details | 5DAI | | 10DAI | | 15DAI | | 20DAI | | 25DAI | | 30DAI | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *PDS | Per cent reduction over control | *PDS | Per cent reduction over control | *PDS | Per cent reduction over control | *PDS | Per cent reduction over control | *PDS | Per cent reduction over control | *PDS | Per cent reduction over control |
| $T_1$ | EB-12 | 17.00$^{abc}$ | 47.42 | 22.33$^{bc}$ | 41.23 | 25.33$^{bcd}$ | 44.52 | 28.67$^{bc}$ | 48.50 | 30.67$^{abc}$ | 48.87 | 36.00$^{b}$ | 46.54 |
| $T_2$ | EB-13 | 21.33$^{cd}$ | 34.02 | 25.33$^{c}$ | 33.33 | 30.67$^{e}$ | 32.85 | 33.67$^{d}$ | 39.52 | 37.00$^{de}$ | 38.33 | 43.33$^{c}$ | 35.64 |
| $T_3$ | EB-15 | 12.40$^{a}$ | 61.65 | 16.20$^{a}$ | 57.37 | 20.00$^{a}$ | 56.20 | 21.80$^{a}$ | 60.84 | 26.60$^{a}$ | 55.66 | 29.60$^{a}$ | 56.04 |
| $T_4$ | EB-26 | 22.33$^{d}$ | 30.92 | 26.33$^{c}$ | 30.70 | 29.66$^{de}$ | 35.04 | 34.67$^{d}$ | 37.73 | 41.00$^{e}$ | 31.66 | 45.33$^{c}$ | 33.67 |
| $T_5$ | EB-31 | 15.40$^{ab}$ | 52.37 | 20.00$^{ab}$ | 47.37 | 23.80$^{abc}$ | 47.88 | 26.60$^{b}$ | 52.21 | 29.60$^{abc}$ | 50.66 | 31.40$^{a}$ | 53.37 |
| $T_6$ | *Pf* (KAU) | 19.00$^{bcd}$ | 41.23 | 22.00$^{bc}$ | 42.11 | 26.00$^{bcd}$ | 43.06 | 31.00$^{cd}$ | 44.31 | 32.00$^{bcd}$ | 46.66 | 36.00$^{b}$ | 46.54 |
| $T_7$ | Streptocyclin e (300 ppm) | 13.40$^{a}$ | 58.55 | 19.00$^{ab}$ | 50.00 | 22.00$^{a}$ | 51.82 | 25.00$^{ab}$ | 55.10 | 28.00$^{ab}$ | 53.33 | 31.00$^{a}$ | 53.96 |
| $T_8$ | Turmeric + sodium bicarbonate mixture (10:1) | 20.33$^{cd}$ | 37.11 | 23.33$^{bc}$ | 38.59 | 27.67$^{cde}$ | 29.42 | 30.67$^{cd}$ | 44.91 | 34.00$^{cd}$ | 43.33 | 38.00$^{b}$ | 43.50 |
| $T_9$ | Control | 32.00$^{c}$ | | 38.00$^{d}$ | | 45.67$^{f}$ | | 55.67$^{e}$ | | 60.00$^{f}$ | | 67.33$^{d}$ | |

In each column figures followed by same alphabet do not differ significantly according to DMRT

* Mean of five replications.

PDS: Per cent disease severity. DAI: Days after inoculation. .

**Fig.4.4: Effect of endophytes on the severity of bacterial blight of anthurium**



other. On 10 days and 15 days after inoculation also $T_3$ was the best and was on par with $T_5$ and $T_7$. However, observation on 20 days after inoculation showed that, $T_3$ (EB-15) was the best in reducing the disease severity.

It was on par with only $T_7$ (Streptocycline). Later at 25 days after inoculation, $T_3$ was leading and was on par with $T_5$, $T_7$ and $T_1$. On the last day of observation (30 days after inoculation), $T_3$ was followed by $T_7$ and $T_5$ respectively and they were on par with $T_3$. During the study, $T_9$ (control) showed disease severity ranging from 32-67.3 per cent which was the highest among all treatments. After five and ten days after inoculation

T6 (*P. fluorescens*) recorded a PDS of 19.00 and 22 respectively which was on par with $T_8$ (Turmeric sodium bicarbonate mixture). Fifteen days after inoculation $T_6$ recorded a PDS of 26 which was on par with $T_1$. After 20, 25 and 30 days after inoculation it recorded a PDS of 31.00, 32.00 and 36.00 respectively which was on par with. $T_8$ (Turmeric-sodium bicarbonate mixture).

**4.12. Characterization of endophytes**

The cultural, morphological and biochemical characters of the two promising endophytes were studied. EB-15 was gram positive while the other one (EB-31) was gram negative. The colonies of EB-15 were dry, white and spreading. EB-31 produced smooth - slimy fluidal colonies. Both the isolates produced water soluble pigments which showed fluorescence under UV light.

They showed positive reaction for production of catalase, oxidase, arginine dihydrolase, starch hydrolysis and utilized citrate as sole source of carbon. However they are negative with regard to nitrate and indole production test. None of the isolates produced Hydrogen sulphide. They showed lipolytic activity.

Among carbon compounds tested all isolates utilized acid in glucose dextrose, maltose, lactose, fructose, mannose, mannitol and sucrose. They were unable to utilize arabinose, sorbitol, inositol, cellobiose, adinitol, glycerol and

dulcitol. Based on cultural, morphological and biochemical characters EB-15 was tentatively identified as *Bacillus* sp. whereas EB-31 *Pseudomonas* sp.

# *DISCUSSION*

# 5. DISCUSSION

Anthurium is an important cut flower crop of Kerala. It has got good market within and outside the State. Bacterial blight caused by *Xanthomonas axonopodis* pv *dieffenbachiae* is one of the major constraints in anthurium cultivation. This disease was reported from Kauai Islands, Hawaii in early 1970s In India, the occurrence of the same was reported in imported anthurium plants by Satyanarayana *et al*. (1998).

Although application of antibiotics may lessen the incidence and severity of the bacterial diseases, it cannot be continued as long term solution because of the chance of development of resistant strains of the pathogen (Anjana, 2005). Nowadays more emphasis is being given on biological management of plant diseases. Biocontrol agents isolated from rhizosphere and phylloplane have been extensively studied for the management of plant disease. But their success greatly depends on the host, environment and many other conditions. These problems can be overcome to a great extent by use of antagonistic endophytes, since the internal habitat ensure supply of nutrients and protects them from competition with other microbes (Manjula *et al*., 2002) A perusal of the literature reveals that no attempts have been made so far from Kerala for the management of bacterial blight of anthurium using endophytic microorganisms. Thus the present investigation is focused to harness the potential effect of endophytic microbes on the management of the dreaded bacterial blight of anthurium caused by *Xanthomonas axonopodis* pv. *dieffenbachiae.*

## 5.1 Symptomatology

For better understanding of the disease and its correct identification, it is essential to have a clear cut idea about the symptomatology. Therefore the symptomatology of disease was studied in detail under natural and artificial condition. Naturally infected plants showed a variety of symptoms. The initial symptom appeared as irregular water soaked lesion on leaf margin and lamina.

They increased in size and turned dark brown with a prominent yellow halo. When considerable portion of lamina get blighted leaves shed. Symptoms were also present on spathe as well as on spadix. On spathe instead of a yellow halo brownish black lesions with wavy fringed margin were formed. Affected spadix finally turned to a black candle like structure. These symptoms have been reported by Hayward (1972) with slight variations. Systemic infection started as water soaked lesion at collar region of plant leading to rotting. Leaves showed browning and yellowing. Infection spread from collar region through the petiole to the leaf base and the leaf lamina. In about 2-3 weeks after appearance of the first symptom the whole plant dried. The systemic phase of disease has been described by many workers (Chase *et al*., 1992; Das *et al*., 1996 and Dhanya, 2000) and this phase of infection causes serious concern to anthurium growers as it leads to complete death of plant. In addition, systemic infection helped to spread the pathogen to other places when apparently healthy suckers from infected plants are used for planting.

**5.2 Isolation and characterization of the pathogen**

The bacterium causing bacterial blight of anthurium was isolated from diseased leaves, pathogenicity established and pure culture was maintained in Potato Sucrose Peptone Agar slants. Morphological, cultural and physiological characters of pathogen were studied in detail. The bacterium was aerobic, gram negative rods and produced yellow circular, convex colonies with entire margin. It produced water insoluble yellow pigment. The observations were in confirmity with Hayward (1972). It utilized glucose oxidatively, hydrolyzed starch, liquefied gelatin and produced $H_2S$ and ammonia. Tests for indole, Nitrate reduction, MR test and growth at 6 per cent NaCl were negative. The bacterium utilized glucose, maltose, lactose, fructose, dextrose, sucrose, mannose and arabinose and did not growing media containing sorbitol, inositol, cellobiose, adinitol, glycerol, dulcitol and mannitol. Thus, based on cultural, morphological and biochemical characters coupled with pathogenicity, the bacterium causing bacterial blight of anthurium is

tentatively identified as *Xanthomonas axonopodis* pv. *dieffenbachiae* in line with those reported by Pohronezny *et al*. (1985), Alvarez *et al*. (1990) and Dhanya (2000).

**5.2 Isolation and enumeration of endophytic microbes**

Biocontrol agents isolated from rhizosphere and phylloplane in many cases showed inconsistent performance due to their dependence on environmental conditions. This problem can be overcome by endophytes by way of internal habitat, assured nutrient supply and protected environmental conditions. Hence, attempts were made to select endophytes from healthy anthurium plants collected from various locations. It is established that the total number of endophytes that invade plant is controlled by plant type and environmental conditions. Isolation of endophytic microbes yielded more number of bacteria than fungi. Further there was variation in the population of endophytes in samples collected from different locations. This result is in line with earlier reports by Kloepper *et al.* (1980) and Fisher *et al*. (1992). According to Hallmann *et al*. (1997), plant and soil factors especially pH, affect endophytic microbes indirectly by altering saprophytic community in rhizosphere thus preselecting potential endophytes. Out of the 51 representative endophytes selected for further studies 37 were bacterial and rest were fungal endophytes. It was also noticed that, root portion yielded more endophytes compared to leaf and petiole, which is in agreement with the reports of many other workers (Tripathi *et al.,* 2006; Rajendran *et al*., 2006; Saisree, 2007 and Aravind *et al.,* 2009).

**5.3 *In vitro* evaluation of endophytes for their antagonistic effect against the pathogen**

Selection and identification of growth promoting and disease suppressive biologically efficient microbes through *in vitro* and *in vivo* assay is crucial and is the first step towards the development of an effective biocontrol agent before

launching its field evaluation (Weller *et al*., 1988). Thus, it is pertinent that the efficacy of such microbes should be established under lab conditions and the most effective ones are selected for further pot/ field evaluations. Hence, the *in vitro* antagonistic effect of the isolated endophytes against *Xanthomonas axonopodis* pv. *dieffenbachiae* were tested in comparison with the standard cultures of fungal (*T. harzianu*m IISR and *T. viride*) and bacterial (*P. fluorescens* from TNAU and KAU) bioagents. Of the 14 endophytic fungi selected only two (EF-2, EF-5) showed slight zone of inhibition. Thus they were not selected for further studies. Among the 37 endophytic bacteria screened, most of the isolates were found to be ineffective and only eight were beneficial and exhibited antagonistic activity against the pathogen. The highest inhibition of the growth of pathogen was observed with the endophytic bacterial isolates, EB -15 and EB-31. The inhibitory action may be due to production of secondary metabolites and siderophores by the bacterial endophytes. Such inhibitory effect of endophytic microbes against bacterial and fungal pathogens was reported by Rahman and Khan (2002), Bacon and Hinton (2002),Liu *et al*. (2003) Ji *et al*.(2008)and Muthukumar (2008).

### 5.4 Mechanism of antagonism

Endophytic bacterial isolates which showed antagonistic effect were further subjected to various tests for understanding the parameters that contribute to antibacterial action. Consequently, they were tested for their growth promoting action, production of hydrogen cyanide, ammonia, growth regulators and siderophores and also their effect on P solubililzation in comparison with reference strain of *Pseudomonas fluorescens* (KAU).

#### 5.4.1 Vigour Index

The eight endophytes along with reference culture of *P. fluorescens* (KAU) were bio assayed for their ability to promote or inhibit growth of sorghum seeds. In general, seeds bacterized with endophytes exhibited better germination per cent thereby indicating that the endophytes have the potential to enhance plant growth.

Such growth promoting effects of endophytes in various crops have been reported by many workers. (Sturz *et al*., 1998; Sharma and Nowak,1998;Varma *et al*., 1999; Nejad and Johnson, 2000;Surette *et al*.,2003; Kulinseky *et al*., 2004 and Vijayaraghavan, 2007).

### 5.4.2 Production of HCN

With regard to the production of HCN, it was seen that two of them *viz.,* EB-14 and EB-31 were cyanogenic. Rest of the isolates were non cyanogenic when tested with supplemented King's B medium. Bano and Mussarrat (2003) noticed low HCN Production under iron limiting conditions. Production of HCN by endophytes in various crops is well documented (Nejad and Johnson, 2000 and Paul, 2004).

### 5.4.3 Production of ammonia

The ability of endophytes for the producting of ammonia, a volatile compound having direct effect on biocontrol activity, is well documented (Paul, 2004). The present study revealed that all the selected endophytes exhibited varying levels of ammonia production. The isolates (EB-15 and EB-31) which gave the highest inhibitory effect against the pathogen showed the highest level of ammonia production also. The production of ammonia by bacterial biocontrol agents in suppressing plant pathogens, triggering growth promotion and induction of systemic resistance in host plants have been documented (Ryu *et al.,* 2003).

### 5.4.4 Phosphorous (P) solubilization

One of the important criteria to assess the efficiency of plant growth promoting bacteria is their ability to transform unavailable 'P' to available form. So in the present study, an attempt was made to assess P solubilizing capacity of selected endophytes along with reference culture of *P. fluorescens* (KAU). Among the eight isolates, only 4 *viz.*, EB-15, EB-31 EB-12 and EB-8 showed P

solubilisation ability on Pikokvsya's medium. The maximum P solubilization was with EB-15. Such ability of endophytes in solubilizing P has been well documented (Parihar *et al*., 2004 and Kulinsky *et al.,* 2004). Vijayaraghavan (2007) noticed P solubilization capacity of PGPR isolates of ginger. Further, conferring resistance to plants to stress conditions by mobilizing P for plant growth was reported.

### 5.4.5 Assay of growth promoting hormone

It is well established that the application of bio agents has a positive effect in promoting growth of plants. This effect is attributed to the production of phytohormones like auxin, gibberilin and cytokinins by bioagents. Among this IAA plays an important role in growth promotion. Production of IAA by different endophytic microbes from various crops has been reported. (Holland, 1997; Tripathi *et al*., 2006; Saisree 2007 and Vijayaraghavan, 2007). Hence, in this study the capacity of eight endophytes in IAA production was assessed. All the isolates produced IAA ranging from 7-200 µg $ml^{-1}$. The highest IAA production was noticed in case of EB-15. This was reflected in the vigour index of seedlings treated with this isolate.

## 5.5 Detection of siderophores

Bacteria under iron limiting conditions produce siderophores to escape from deleterious effects due to unavailability of iron. They have evolved mechanisms which under iron limiting condition selectively chelate iron for their own purpose and make it unavailable to others. Hence, the production of siderophore is correlated with the antagonistic potential. So the potential antagonistic endophytes selected were tested for their capacity to produce siderophores. All the isolates except EB-12 produced siderophores as evidenced by their fluorescence. This implies that EB-12 is less competitive than other endophytes and may explain the weak antibiosis of this isolate compared to the other siderophore producing endophytes. Similar findings of siderophore

production were documented by many workers (Kloepper *et al*., 1980; Banu and Mussarrat, 2003; Paul, 2004; Tripathi *et al*., 2006 and Vijayaraghavan, 2007). The study also revealed that the capacity of the isolates to produce siderophores reduced as the concentration of $FeCl_3$ is increased.

### 5.6 Compatibility of selected endophytes to plant protection chemicals and fertilizers

Anthurium being an important cut flower crop, growers are taking all measures to protect the plant from pests and diseases. Even after adopting to various cultural and chemical means, bacterial blight of anthuruim continues to be difficult to manage. While adopting integrated disease management practice using antagonist, it is imperative that pesticides including fungicides, insecticides antibiotics and even fertilizers commonly used in anthurium must be compatible with biocontrol agents and further care must be taken to select suitable combination. In this context, attempts were made to elucidate the compatibility of selected endophytic isolates with fungicides, insecticides, antibiotics and fertilizers.

#### 5.6.1 Antibiotics

The antibiotic sensitivity of promising endophytes along with reference culture was tested. In general the isolates showed varying levels of sensitivity towards the antibiotics. Isolates EB-14 and EB-32 were found compatible with Penicillin G and Tetracycline whereas B-13 was found compatible with Penicillin. All other antibiotics tested showed inhibition indicating their incompatibility to various extends with Gentamycin showing the maximum incompatibility. Such variation in sensitivity of PGPR isolates to antibiotics was reported by Vijayaraghavan (2007).
Since the proprietary antibiotic Streptocycline is widely recommended for the management of bacterial blight of anthurium, the compatibility of this

antibiotic with the bacterial endophytes were also tested so as to know whether this chemical can be combined with the endophytes in the integrated management of the disease. But all the isolate were found sensitive to Streptocycline to varying extent with EB-8 showing maximum sensitivity. Higher level of sensitivity of endophytes to antibiotics, compared to rhizosphere bacteria has been reported earlier by Rangeshwaren and Kumar (2008).

### 5.6.2 Fungicides

A total of six fungicides were tested to study the compatibility of these chemicals to the selected endophytes along with the reference strains. It was observed that Bavistin, Akomin and Contaf at different concentrations tested were compatible with all isolates. All other fungicides tested *viz.,* Mancozeb, Captaf and Saaf showed inhibition indicating incompatibility to various extends with Mancozeb showing maximum incompatibility which was on par with Saaf. A perusal of literature revealed no reports on the compatibility of fungicides with endophytic bacteria from anthurium. The compatibility of Bavistin, Akomin amd Mancozeb with antagonistic rhizosphere and phyllosphere microflora was reported (Mathew, 2003; Bhavani, 2004 and Vijayaraghavan, 2007). Contrary to this, the endophytes tested in this study were incompatible with Mancozeb, a fungicide regularly used by anthurium growers. Hence, utmost care should be taken in this regard and also with many other incompatible fungicides in the IDM of anthurium.

### 5.6.3 Insecticides

In this study none of the insecticides *viz.,* Ekalux, Monocrotophos, Malathion, Rogor, Classic 20 and Hostathion tested inhibited the growth of the isolates revealing their compatibility. Similar results were documented by Bhavani (2004) and Vijayaraghavan (2007). Hence, these insecticides can be safely applied for pest management without any deleterious effect to the endophytes.

### 5.6.4 Fertilizers

It is always desirable to use boicontrol agents which are compatible with recommended fertilizers especially in the plant like anthurium where foliar applications of fertilizers are regularly practiced. Hence, the compatibility of Urea, Rajphos, MOP and Factomphos with selected endophytes was ascertained and it was found that all of them were compatible with the isolates indicating their tolerance to the same. The compatibility of endophytes from anthurium with fertilizers has not been studied well. However, similar works were reported with antagonistic phylloplane and rhizosphere bacteria isolated from cocoa (Bhavani, 2004 and Vijayaraghavan, 2007).

### 5.7 *In vitro* sensitivity of pathogen to plant protection chemicals.

Another study was conducted to find out the *in vitro* inhibitory effect of antibiotic, fungicide and turmeric sodium bicarbonate mixture commonly used in anthurium on the growth of *Xanthomonas axonopodis* pv. *dieffenbachiae.* Streptocycline, Captaf, Mancozeb and Turmeric-sodium bicarbonate mixture was used for *in vitro* testing. Streptocycline showed maximum inhibition followed by Mancozeb which was on par with Turmeric sodium bicarbonate mixture. Effect of turmeric sodium bicarbonate mixture against pathogen under *in vitro* and *in vivo* conditions was noticed by Dhanya (2000). Inhibitory effect of Streptocycline towards *Xanthomonas axonopodis* pv. *dieffenbachiae* was documented by many workers (Dhanya, 2000 and Valsalakumari and Rajeevan, 2007).

### 5.8 Mutual compatibility of selected endophytes

Eventhough, considerable progress in the development of effective bio control agent have been made over a period of time,application these organism at times may not give the expected results owing to failure in its adaptability to

various ecological conditions. Hence, nowadays focus is being made to develop consortia of useful bioagents selected from different areas with more adaptability to the different ecological niche to obtain maximum effect. In this context, mutual compatibility of effective endophytes was studied. It was observed that, all the eight isolates were compatible to each other in the *in vitro* study indicating the possibility of using these isolates as consortia for the management of the disease. There are several reports of biocontrol agents used in combination for plant disease management and most of these reports showed that combining antagonists resulted in improved bio control (Mazzola *et al.,* 1995 and DeBoar *et al.,* 1997). Fukui *et al.* (1999) reported the successful suppression of *Xanthomonas axonopodis* pv. *dieffenbachiae* using non pathogenic bacteria and bacterial mixtures.

**5.9 Pot culture evaluation of potential endophytes against bacterial blight pathogen**

After evaluating the *in vitro* inhibitory effect against the pathogen by the selected endophytic bacteria of anthurium, a pot culture experiment was conducted to assess their field efficacy. For this, five out of the selected eight endophytes were used based on their inhibitory effect against the pathogen. In addition, their effects were compared with reference culture of *P. fluorescens,* antibiotic Streptocycline and turmeric-sodium bicarbonate mixture. The pathogen was artificially inoculated after two pre-inoculation sprays with endophytes and other chemicals. Further, two more sprays were given. Observations on the incidence and severity of the disease were taken at five days interval after inoculation of pathogen for 30 days.

In the initial stage of the experiment, lowest PDI was observed for treatment EB-15 which differed significantly from all other treatments. Later, all the treatments recorded cent per cent disease incidence. From the data of PDS taken on five days after first spray it was revealed that various treatment differ significantly among themselves and in plants sprayed with EB 15 recorded lowest

PDS with 61.65 per cent reduction over control which was par with $T_7$ (Streptocycline) and $T_5$ (EB-31). Different endophytes showed a range of 30.92 to 61.65 per cent reduction of disease over control. After 10, 15, 20 and 25 days, after second spraying also treatment $T_3$ (EB-15) recorded minimum PDS. After 30 days, maximum PDS was noticed in case of $T_9$ (67.33) followed by $T_4$ (45.33). The standard culture of *Pseudomonas fluorescen*s showed reduction in disease severity in a range of 41.23 - 46.54. Turmeric-sodium bicarbonate mixture showed reduction in disease severity in a range of 37.11- 43.50. Contradictory to the findings of Dhanya (2000), it was observed that turmeric-sodium bicarbonate was not much efficient in managing the disease. Continuous five rounds spraying might be essential for proper management of the disease.

Thus the study revealed that, bacterial endophytes are capable of inhibiting the bacterial blight pathogen of anthurium. Among the effective ones, isolates EB-15 and EB-31 were more promising. They were more efficient than standard culture of *P. fluorescens* (KAU). In the pot culture experiment also, isolate EB-15 exhibited more efficiency in managing bacterial blight disease than recommended antibiotic Streptocycline. EB-31 was also found equally effective and good . Hence, the most effective endophytes can be used to contain the disease in anthurium. Efficiency of the endophytes might be due to competition, antibiosis, production of secondary metabolites or due to induction of systemic resistance. A perusal of literature revealed no works on effectiveness of the endophytes in managing bacterial blight of anthurium. There are several reports which emphasize the efficiency of endophytic bacteria in reducing disease severity of other crop plants. Pleban *et al.* (1995) reported the usefulness of endophyte *B. subtilis, B cerens* and *B. pumilis* in reducing disease in bean seedlings. Ziedan (2006) observed the control of root and pod rot disease of peanut caused by *Aspergillus niger* and *Fusarium oxysporum* by endophytic bacteria *B. subtilis* and *P fluorescens*. Rahman and Khan (2002), Liu *et al*. (2003) Li *et al.*(2008), Muthukumar (2008) and Nara *et al*.(2008) observed the control of wilt of solanaceous vegetables using endophytic microbes. Management of Black shank of tobacco,Quick wilt of pepper and Basal stem rot of coconut using antagonistic

endophytes were reported by Wang and Xiao (2003),Aravind *et al*. (2009) and Zaiton *et al*. (2008) respectively.

**5.10 Characterization of promising endophytes**

Attempts to identify the two most promising endophytes (EB-15 and EB-31) were carried out by studying the cultural, morphological and biochemical characters. It was observed that, EB-15 was Gram positive and EB-31 was Gram negative. Based on cultural, morphological and biochemical characters studied and as described by Harrigan and McCane (1966) and Bergy's Manual of Systematic Bacteriology, Vol. I (Staley *et al*., 1989), EB-15 was tentatively identified as *Bacillus* sp. and the other isolate as *Pseudomonas* sp.

Thus the present study throws light on the potentiality of using endophytic bacteria for the management of bacterial blight of anthurium. Among the endophytes used EB-15, identified as *Bacillus* sp. performed very well both under *in vitro* and *in vivo* conditions. The significant reduction of bacterial blight incidence by endophytes especially by EB-15 may be due to the production of inhibitory substances like ammonia, siderophores, antibiotics etc. However, investigations on the potential of the isolate in different agro climatic situation need to be ascertained.

# *SUMMARY*

## 6. SUMMARY

One of the major constraint in anthurium cut flower production is bacterial blight incited by *Xanthomonas axonopodis* pv. *dieffenbachiae*. It causes considerable economic loss. Considering the serious nature of the disease the present investigation was undertaken to harness the potential of antagonistic endophytes against bacterial blight pathogen.

The bacterial blight pathogen was isolated from infected leaves and purified by repeated streaking on Potato Sucrose Peptone Agar (PSPA) medium. The bacteria produced slimy yellow colonies and were aerobic gram negative rods. It produced acid oxidatively from sucrose, glucose, maltose, lactose, dextrose, fructose, sucrose, mannose and arabinose whereas no acid was produced from dulcitol, inositol, adinitol and sorbitol. It produced $H_2S$, liquefied gelatin and hydrolyzed starch. It utilized citrate, produced lipase and ammonia whereas it showed negative reaction for methyl red and urease test. Based on morphological, physiological and biochemical characters coupled with pathogenicity the pathogen was identified as *Xanthomonas axonopodis* pv. *dieffenbachiae*

Symptoms of disease both under natural and artificial conditions were studied. The typical symptoms such as initial water soaked lesions, development of necrotic area surrounded by yellow halo and flower and petiole infection were observed. In severe case pathogen become systemic in nature. All the general symptoms of naturally infected plants could be reproduced on artificial inoculation.

Endophytic microorganisms were isolated from healthy anthurium plants collected from different locations. A quantitative estimation of endophytic micro organisms revealed the abundance of bacteria than fungi. Moreover, more number of endophyte were isolated from roots than from petiole and leaves. Based on

cultural characters of endophytic microorganisms, 14 fungal and 37 bacterial endophytes were selected. Antagonistic action of these endophytes against pathogen was studied in comparison with that of reference culture of *Pseudomonas fluorescens* (KAU), *P. fluorescens* (TNAU), *Trichoderma viride* (KAU) and *T. harzianum* (IISR). The study revealed that, only eight bacterial and two fungal cultures showed antagonistic action against the pathogen. Since fungal endophytes showed less inhibition,they were not selected for further studies.

The selected endophytes were subjected to various tests for understanding parameters that may act to produce antagonism as well as enhanced growth of the plants. All the antagonists inhibited growth of the pathogen, promoted plant growth, produced IAA and ammonia. Only two isolates *viz.,* EB-14 and EB-31 are found cyanogenic. Only four isolates *viz.,* EB-15, EB-31, EB-26 and EB-12 were found capable of solubilizing phosphate. All isolates except EB-12 were found capable of producing siderophores.

The compatibility of endophytes with antibiotics, fungicides and fertilizers were tested. The isolates showed variations in their compatibility to antibiotics, tested. Among fungicides, Bavistin, Contaf and Akomin were compatible with all endophytes, while they were incompatible with Mancozeb, Saaf and Captaf. Isolates were slightly sensitive to turmeric sodium bicarbonate mixture. All isolates were compatible with all insecticides and fertilizers tested. Further they were compatible with each other. The pathogen was highly sensitive to Streptocycline, Mancozeb and Captaf and Turmeric sodium bicarbonate mixture.

Based on the study five endophytes were selected for evaluating their field efficacy. For this a pot culture experiment was conducted. Two pre inoculation sprays with endophytes were given at five days interval before challenge inoculation of the pathogen. Further, two more sprays were given and observations were recorded up to 30 days at five days interval. Considerable

reduction in disease incidence and severity was noted in plants treated with endophytes. In initial stage of experiment lowest PDI was observed in plants treated with EB-15 ($T_3$) which differed significantly from all other treatments. There was significant difference among treatments in reducing disease severity. Among the treatment the maximum effect in reducing the disease was with endophytic bacterial treatment EB-15 followed by treatments $T_7$ (Streptocycline) and $T_5$ (EB-31).

Attempts to identify the most effective endophytes *viz.,* EB-15 and EB-31 were done by studying their cultural, morphological and biochemical characters, Based on the results the bacteria were tentatively identified as *Bacillus* sp. (EB-15) and *Pseudomonas* sp. (EB-31).

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# *APPENDIX-1*

## APPENDIX-1

## Media Composition

(Ingredients per litre)

### 1. Potato Sucrose Peptone Agar media (PSPA) (pH 7.0)

| | | |
|---|---|---|
| $KH_2PO_4$ | : | 0.2 g |
| $Na_2HPO_4$ | : | 0.5 g |
| $Ca(NO_3)_2$ | : | 0..5 g |
| $FeSO_4$ | : | 0.05 g |
| KCl | : | 0.05 g |
| Peptone | : | 2.0 g |
| Sucrose | : | 20.0 g |
| Potato | : | 300.0 g |
| Agar | : | 20.0g |
| Distilled water | : | 1000 ml |

### 2. Basal medium (pH 7.0)

| | | |
|---|---|---|
| Peptone | : | 1.0 g |
| $NH_4H_2PO_4$ | : | 1.0 g |
| KCl | : | 0.2 g |
| $MgSO_4$ | : | 0.2 g |
| Bromothymol blue | : | 0.03 g |
| Agar | : | 3.0 g |
| Distilled water | : | 1000 ml |

### 3. Methyl red broth (pH 7.0)

| | | |
|---|---|---|
| Peptone | : | 5.0 g |
| Glucose | : | 5.0 g |
| $KH_2PO_4$ | : | 0.2 g |
| Distilled water | : | 1000 ml |

### 4. Yeast Glucose Chalk Agar media (YGCA) (pH 7.0)

| | | |
|---|---|---|
| Yeast extract | : | 10.0 g |
| Glucose | : | 10.0 g |
| Chalk($CaCO_3$) | : | 20.0 g |
| Agar | : | 20.0 g |
| Distilled water | : | 1000 ml |

### 5. Kings'B media (pH 7.2)

| | | |
|---|---|---|
| Peptone | : | 20.0 g |
| Glycerol | : | 10.0 ml |

| | | |
|---|---|---|
| $K_2HPO_4$ | : | 1.5 g |
| $MgSO_4$ 7 $H_2O$ | : | 1.5g |
| Agar | : | 20.0 g |
| Distilled water | : | 1000 ml |

**6. Peptone water (pH 7.0)**

| | | |
|---|---|---|
| Peptone | : | 10.0 g |
| NaCl | : | 5.0 g |
| Distilled water | : | 1000 ml |

**7. Pikovaskya's medium (pH 7.0)**

| | | |
|---|---|---|
| Glucose | : | 10.0 g |
| $Ca(PO_4)_3$ | : | 5.0 g |
| $NH_4SO_4$ | : | 0.5 g |
| KCl | : | 0.23 g |
| $MgSO_4$ | : | 0.1 g |
| $MnSO_4$ | : | trace |
| $FeSO_4$ | : | trace |
| Yeast extract | : | 0.5 g |
| Agar | : | 20.0 g |
| Distilled water | : | 1000 ml |

**8. Luria Bertani broth (LB) (pH 7.2)**

| | | |
|---|---|---|
| Tryptone | : | 10.0 g |
| Yeast extract | : | 5.0 g |
| Glucose | : | 1.0 g |
| NaCl | : | 10.0 g |
| Distilled water | : | 1000 ml |

**9. Nutrient Agar media (NA) (pH 7.2)**

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

**10. Thronley's media (pH 7.2)**

| | | |
|---|---|---|
| Peptone | : | 1.0 g |
| NaCl | : | 5.0 g |
| $K_2HPO_4$ | : | 0.3 g |
| Agar | : | 3.0 g |
| Phenol red | : | 0.01 g |
| L arginine | : | 1.0 g |
| Distilled water | : | 1000 ml |

**11. Nutrient Gelatin media (pH 7.2)**

| | | |
|---|---|---|
| Peptone | : | 10.0 g |
| Beef extract | : | 5.0 g |
| Gelatin | : | 4.0 g |
| Agar | : | 20.0 g |
| Distilled water | : | 1000 ml |

**12. Sierra's media (pH 7.0)**

| | | |
|---|---|---|
| Peptone | : | 10.0 g |
| NaCl | : | 5.0 g |
| $CaCl_2$ 1 $H_2O$ | : | 0.1g |
| Agar | : | 20.0 g |
| Distilled water | : | 1000 ml |

**13. Simmon's Citrate Agar (pH 7.0)**

| | | |
|---|---|---|
| **N**$H_4H_2PO_4$ | : | 1.0g |
| $KH_2PO_4$ | : | 1.0g |
| NaCl | : | 5.0g |
| Sodium citrate | : | 2.0g |
| $MgSO_4$ | : | 0.2g |
| Agar | : | 15.0g |
| Bromothymol | : | 0.08g |

**14. Vanden Mooter Succinate Medium (pH 6-7.0)**

| | | |
|---|---|---|
| $K_2HPO_4$ | : | 0.5g |
| $KH_2PO_4$ | : | 0.5g |
| $K_2SO_4.7H_2O$ | : | 0.2g |
| Sodium succinate | : | 2.0g |
| $KNO_3$ | : | 3.0g |
| Yeast extract | : | 5.0g |
| Agar | : | 3.0g |

**15. Tryptophan Broth (pH 7.0)**

| | | |
|---|---|---|
| Tryptophan | : | 10.0g |
| NaCl | : | 5.0g |
| Water | : | 1000ml |

**16. Christensen's Media (pH 7.0)**

| | | |
|---|---|---|
| Peptone | : | 1.0g |
| Glucose | : | 1.0g |
| NaCl | : | 5.0g |
| $KH_2PO_4$ | : | 2.0g |
| Phenol red | : | 0.1g |
| Agar | : | 20.0g |
| Distilled water | : | 1000ml |

# *ABSTRACT*

# POTENTIAL OF ANTAGONISTIC ENDOPHYTES AGAINST BACTERIAL BLIGHT OF ANTHURIUM

**By**

**Sanju Balan**

**ABSTRACT OF THE THESIS**

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## ABSTRACT

One of the major constraints in anthurium cultivation is the severe incidence of bacterial blight disease. The pathogen causing bacterial blight of anthurium was isolated and identified as *Xanthomonas axonopodis* pv. *dieffenbachiae* based on its cultural, morphological and biochemical characters coupled with its pathogenicity. Endophytic microbes were isolated from different parts of healthy anthurium collected from various locations of Thrissur, Kannur, Kasargod and Thiruvananthapuram districts. Isolation yielded more number of bacteria than fungi. Out of 51 endophytes tested, only eight bacterial and two fungal isolates showed antagonism against the pathogen.

The eight selected bacterial endophytes were subjected to various tests for understanding the parameters that may act to produce antagonism as well as enhanced growth of the plants. The antagonists varied in their ability to promote plant vigour, hydrogen cyanide, IAA, ammonia and siderophore production and Phosphorus solubilization capacity.

The endophytes were compatible with Bavistin, Akomin and Contaf and incompatible with Indofil M 45, Saaf and Captaf. Six insecticides *viz.,* Classic, Rogor, Ekalux, Malathion, Target and Hostathion, and four fertilizers *viz*. Muriate of potash, Rajphos, Urea and Factomphos were compatible with the isolates. However, the isolates exhibited variation in their sensitivity with the antibiotics tested. Further, all isolates were compatible to each other.

A pot culture experiment was conducted to assess the field efficacy of selected endophytes in comparison with recommended management practices. The treatments were given as two pre inoculation and two post inoculation sprays. The incidence and severity of the disease were recorded at five days interval for a period of 30 days. The result indicated that plants treated with endophyte EB15 showed minimum disease incidence and severity. This was followed by treatment with EB 31 and Streptocycline. The promising endophytes, EB15 and EB 31 were tentatively identified as *Bacillus* sp. and *Pseudomonas* sp. respectively.