ENDOPHYTIC MICROORGANISM MEDIATED SYSTEMIC RESISTANCE IN COCOA AGAINST *Phytophthora palmivora* (Butler) Butler

By

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THESIS

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COLLEGE OF HORTICULTURE

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DECLARATION

I, hereby declare that the thesis entitled "**Endophytic microorganism mediated systemic resistance in cocoa against *Phytophthora palmivora* (Butler) Butler**" is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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CERTIFICATE

Certified that the thesis entitled "**Endophytic microorganism mediated systemic resistance in cocoa against *Phytophthora palmivora* (Butler) Butler**" is a record of research work done independently by Ms. Sainamole Kurian, P. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

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Dedicated to my beloved parents

Introduction

1. INTRODUCTION

Cocoa, *Theobroma cacao*, L. belonging to the family Malvaceae has originated in the Amazon basin of South America. The crop prefers a warm humid tropical condition for growth and hence it is confined to the equatorial and tropical countries of the world. The leading cocoa growing countries are Ivory Coast, Ghana, Nigeria and Cameroon, Indonesia, Brazil and Ecuador. According to the latest report by the International Cocoa Organisation, the global production of cocoa beans was 3.5 million tonnes in the 2008/2009 (ICCO, 2010). In India, commercial cultivation of cocoa was started in early 1960s and now it is grown in the southern states of Kerala, Andhra Pradesh, Tamilnadu and Karnataka. Kerala was the leading cocoa growing state till 2004-05 but now, Andhra Pradesh has taken the lead in its cultivation. During 2009-10, Kerala contributed 6344 tonnes of cocoa beans out of the total production of 12954 tonnes in the country compared to 2704 tonnes by Andhra Pradesh (DCCD, 2010).

Since cocoa plant flourishes well under the warm humid tropical climate, the plant is also prone to attack by different pathogens which thrive well under such condition. As such, species of *Phytophthora* attack the crop and cause many diseases inflicting heavy crop losses. Among the various *Phytophthora* diseases, *Phytophthora* pod rot (PPR) is the most serious one and was reported to cause about 44 per cent yield loss. In India, this disease caused by *P. palmivora* was first reported by Ramakrishnan and Thankappan (1965). Later, the involvement of *P. capsici* (Chowdappa, *et al.*, 1993), and *P. citrophthora* (Chowdappa and Chandramohanan, 1996), in the causation of the disease has been reported. However, *P. palmivora* is the ruling causal agent of PPR of cocoa in Kerala. The pathogen is also known to cause canker (Chandramohanan, 1978), seedling dieback (Chandramohanan, 1979), and twig die back and chupon blight (Chandramohanan *et al.*, 1979) in cocoa.

For satisfactory management of PPR, the efficacy of copper based fungicides especially Bordeaux mixture has been well established, provided, its application is coupled with proper cultural practices. However, this disease management strategy often did not give the desired effect due to incorrect preparation and untimely

application of the fungicide. Systemic fungicide metalaxyl and organophosphates like Fosetyl-Al and potassium phosphonate were also used for the management of the disease. But, repeated application of chemical fungicides will give rise to ecological problems including development of resistant strains of the pathogen. Hence, the policy makers, scientists and administrators give much thrust for the development of disease management strategies which are not only safe to the ecosystem but also offer broad spectrum management of plant disease problems through induced systemic resistance in plants. In this context, the role of potential antagonistic microbes which are harmful to the pathogen at the same time beneficial to the plant is of utmost value. Many studies on this line revealed the potentiality of the Plant Growth Promoting Rhizobacteria (Kloepper *et al.*,1980c; Vijayarghavan, 2007), epiphytic (Jimnez *et al.*, 1986) and endophytic microbes (Sturz *et al.*, 1997; Arnold *et al.*, 2003) in the successful management of plant diseases. This line of disease management is well documented in cocoa also (Arnold *et al.*, 2003; Bhavani, 2004). Among the biological control strategies, utilization of potential antagonistic endophytes is considered as a novel approach for efficient disease management due to their intimate systemic association with the plants. The beneficial effects that endophytes can confer on plants have made the study of plant-endophyte associations an important research topic for scientists investigating biological control of diseases in annual, biennial, and perennial crops (Bargabus *et al.*, 2004). Further, endophytes are known to induce systemic resistance (Mishra *et al.*, 2006) in addition to their ability to promote plant growth (Nassar *et al.*, 2005).

The mechanisms involved in the induction of systemic resistance and growth promotion by endophytes have been elucidated in other crops (Benhamou *et al.*, 2000; Rajendran *et al.*, 2006). However a perusal of literature did not reveal many studies related to the role of endophytes especially of those of bacterial origin in the management of PPR of cocoa. So it is imperative to explore possibility of identifying effective antagonistic endophytes from cocoa for the management of the disease. Thus in view of the destructiveness of the disease and to evolve an eco-friendly management strategy to combat PPR, the present investigation was carried out with the following objectives.

1. Isolation and identification of the pathogen from infected cocoa pods.
2. Isolation and enumeration of endophytic microorganisms of cocoa.
3. Assessment of potentiality of antagonistic endophytes against the pathogen.
4. Assessment of growth promoting ability of antagonistic endophytes.
5. Studies on the mode of action and induction of systemic resistance by antagonistic endophytes.
6. Field evaluation of antagonistic endophytes against PPR of cocoa.
7. Characterization and identification of promising endophytes.

Review of literature

2. REVIEW OF LITERATURE

Chocolate has its origins in ancient Central America where the Maya and the Aztecs cultivated the cocoa tree (*Theobroma cacao* L.) and extracted from the seeds a highly prized drink, which was called *chocolati,* a precursor to the word chocolate (Young, 1994). *Theobroma* means the source of the "food of the gods," hence its scientific name *Theo* (god) and *broma* (food). It belongs to the family Malvaceae. Cocoa is a native of Amazon basin of South America, got its entry into India during 1960s. Administratively it is conferred plantation status like coffee, tea and rubber but is seldom recognized as a plantation crop under the Indian Agrarian sector. Cocoa beans are the primary raw material for confectioneries, beverages, chocolates and other edible products. Majority of the processed cocoa products are consumed within India. The tropical diversified congenial climate available in India provides immense scope for its cultivation. Kerala was the principal cocoa growing state in the country accounting for more than fifty per cent of area under cultivation. However, recently Andra Pradesh has taken the lead in area under cocoa cultivation with 16,969 ha compared to 11,044 ha in Kerala. In the last financial year (2009-10) out of the total production of 129,54 tonnes of cocoa beans Kerala contributed 6344 tonnes (DCCD, 2010).

Over the past decades several important fungal diseases have gained considerable importance and pose a serious threat to the supply of chocolate (Keane,1992). *Moniliophthora* pod rot caused by *Moniliophthora roreri* (Cif. and Par.) (Evans *et al.*, 2003), witches' broom, caused by *Crinipellis perniciosa* (Stahel) Singer; and *Phytophthora* pod rot (PPR), caused by *Phytophthora palmivora* (Butl.) Butl. are the most devastating cocoa pod diseases (Wood and Lass, 2001; Bowers *et al.*, 2001). These pathogens are capable of causing complete yield loss. According to Vandervossen, (1997), PPR is the most important disease of cocoa in the world, accounting for as high as 44 per cent of crop loss.

Cocoa is mainly cultivated as an understorey crop in arecanut (*Areca catechu* L.) and coconut (*Cocos nucifera* L.) gardens in India (Chowdappa, *et al.*, 2003) and,

Phytophthora pod rot (PPR) caused by *Phytophthora palmivora* is a major production constraint, causing crop losses ranging from 20 to 30 per cent in the country (ICCO, 2010).

2.1 The pathogen

The first report of the disease was from Guyana and West Indies in 1897 by Jenman and Harrison. Eventhough fungal etiology of the disease was reported as early as in 1898 (Carruthers, 1898), the fungus was identified as belonging to the genus *Phytophthora* only in 1899 (Massee, 1899). Thereafter several species names have been suggested by many workers until Butler (1925) confirmed its identity with that of *Phytophthora palmivora* (Butl.) Butl.

The etiological agent of this disease was identified as a species of *Phytophthora* when it was reported from India (Ramakrishnan, and Thankappan, 1965). It causes various diseases in cocoa other than PPR *viz.*, canker (Chandramohanan,1978), seedling dieback (Chandramohanan,1979), and twig die back and chupon blight (Chandramohanan *et al.*,1979).

In addition to *P. palmivora* which is worldwide in distribution (Gregory, 1974), many other species of *Phytophthora* have been reported from different parts of the world as the causal agent of PPR *viz.*, *P. megakarya* in W. Africa (Griffin, 1977). *P. citrophthora* in Brazil (Campello and Luz, 1981), *P. megasperma* in Venezuela (Zentmyer, 1988) and *P. nicotianae* in Malaysia (Tey and Bong, 1990). *P. capsici* in Central and South America (Zentmyer, 1988).

In India, though *P. palmivora* is the predominant species causing PPR, other species of *Phytophthora viz.*, *P. capsici* (Chowdappa *et al.*, 1993) and *P. citrophthora* (Chowdappa and Chandramohanan, 1996) were also reported.

2.2 Characters of the pathogen

The mycelium of the pathogen is hyaline, coenocytic, (Prem, 1995; Appiah, *et al.*, 2003), measuring 3.22-6.45µm in width (Bhavani, 2004). According to them, the sporangia of *P. palmivora* causing PPR were ellipsoid to ovoid, pappillate and caducous with L/B ratio 1.2-2.2.

Waterhouse (1974) considered pedicel length of sporangia as a stable character under normal conditions. According to Kaosiri *et al.* (1978), caducous sporangia with short stalks produced by cocoa isolates on carrot agar were typical of *P. palmivora*. According to Waterhouse *et al.*, (1983), the size, shape, and length to breadth ratio of sporangia are important characteristics in identification of *Phytophthora* species. Zentmyer (1988) considered sporangial ontogeny as important taxonomic criterion in distinguishing *Phytophthora* spp. and formation of sporangia in *P. palmivora* is in typical sympodial fashion (Brasier and Griffin, 1979; Zentmyer,1988).

Recent taxonomic studies have indicated that the genus *Phytophthora* belongs to class Oomycetes. According to the latest classification of organisms based on molecular phylogeny (Hawksworth *et al.*, 1995), Oomycetes are not included in the Kingdom Fungi. Instead they are considered as a group of 'fungus-like' mycelial organisms that belong to the kingdom Straminopila (Dube, 2005), and represent a unique evolutionary line distant from true fungi. In addition to being dispersed *via* zoospores and generating thick walled sexual oospores, they possess features such as heterokont flagellae (one tinsel and one whiplash) (Barr, 1983), vegetative diploidy (Sansome, 1961, 1965), cellulose in their cell walls (Bartnicki-Garcia, 1987), tubular mitochondrial cristae (Brasier and Hansen. 1992) and in the case of *Phytophthora* spp., lack of epoxidation of squalene to sterols (Griffith and Davis, 1992). All these characters distinguish them from true fungi. Indeed, phylogenetically they are more closely related to the heterokont algae such as the chrysophytes and to diatoms (Sogin and Silberman 1998).

2.3 Symptomatology

Many workers have studied the symptomatology of the *Phytophthora* pod rot of cocoa. Ramakrishnan and Thankappan (1965) reported that cocoa pods are infected by the pathogen at any age *viz.*, from Chirelle to mature. They found that the disease caused a brown discolouration beginning from apical or pedicel end of the pod which spread rapidly and covered the pod surface. A white web of mycelium was also seen on the infected pods, whereby the tissue shrunk and became corky and dark brown in colour. In severe cases, the internal tissues and beans also turned dark brown.

According to Gregory (1974) *P. palmivora* produces other symptoms like seedling blight, trunk canker, twig die back, blight and necrosis of leaves, in addition to rotting of pods. It was also supported by Firman (1974) who found that, *P. palmivora* attacks all parts of the cocoa plant. However, the initiation of the disease as water soaked spots and later turning necrotic is reported by, (Pereira, and Pizzigatti, 1980).

According to Abraham *et al.* (1992), abnormal symptoms were noticed on immature pods infected by *P. palmivora* during rainy season, characterised by concentric rings in the sub-epidermal region of the infected portion of the pod, cementing together of the beans with placenta and husk and watery consistency of the kernel of infected beans.

2.4 Management of the disease

2.4.1 Chemical control

As in the case of any *Phytophthora* disease, copper fungicides were the first choice for the management of PPR. Efficacy of copper fungicides in the management of PPR is well documented *viz.*, Bordeaux mixture (Gorenz, 1971; Menon *et al.*, 1973) Koside (Rocha *et al.*, 1973), Copper oxide and copper oxy chloride (Manalo and Tangonan, 1992). However, according to Chandramohanan (2002) combination of cultural practices and application of Bordeaux mixture was very effective in the management of PPR, of cocoa.

Systemic fungicide, metalaxyl was found effective against *Phytophthora*, as reported by Mc Gregor (1982). Ridomil (metalaxyl) plus Mordox (Cuprous oxide) resulted in substantial reduction in infection by *P. palmivora* on cocoa. Significant reduction in the disease incidence by metalaxyl, chlorothalonil and fentin acetate was reported by Kueh (1984). Spraying with either metalaxyl and copper-1-oxide (Ridomil 72 plus) or cuprous oxide (Nordox 75) was effective against *P. megakarya* on cocoa when appropriate cultural practices are also done (Akrofi and Appiah, 1995).

Phosphonates which do not act directly on the pathogen but stimulate plant defense mechanisms were also used effectively against PPR. Effective and durable control of *Phytophthora* diseases by trunk injection with potassium phosphonate or Aliette (Pegg *et al.*, 1985) and potassium phosphonate (Anderson *et al.*, 1989) were reported whereas, foliar sprays with the same compound was not effective (Holderness, 1990). Guest *et al.* (1994) also reported efficacy of potassium phosphonate as trunk injection in reducing PPR and stem canker of cocoa and in the control of *P. megakarya* and *P. palmivora* (Opoku *et al.*, 1998).

Opoku *et al.* (2007), found that, among fungicides applied as a spray, Ridomil 72 plus at 3.3 g l^{-1} and Kocide DF at 10 g l^{-1} and as injection, 40 ml Foli-R-Fos 400 (commercial formulation of potassium phosphonate) injected twice a year, performed better than the other fungicide treatments in the control of PPR. Recently, potassium phosphonate (Phosphite) applied as trunk injection has been demonstrated to effectively control canker and PPR in Papua New Guinea (Mc Mahon *et al.*,2010).

2.4.2 Biological control

2.4.2.1 Fungal antagonists

Though several antagonistic fungi were reported as efficient in the management of plant diseases, *Trichoderma* occupies a pride of place. Liu and Baker (1980) reported the genus *Trichoderma* as a potential biocontrol agent against fungal pathogens. The efficacy of *T. harzianum* in reducing PPR has been reported by Galindo (1992), and it

was suggested as a potential biocontrol agent to be included in the integrated disease management of PPR of cocoa. Krauss *et al.* (1998) also reported the role of *Trichoderma* against PPR. Five native mycoparasitic strains of *Clonostachys rosea* and three of *Trichoderma* spp. when used together were found effective against PPR (Krauss and Soberanis, 2001). Bhavani, (2004) has reported effective antagonism expressed by epiphytic fungi including *Trichoderma viride* from cocoa pods against *Phytophthora.* Deberdt *et al.*, (2008) reported that, *Trichoderma asperellum* biocontrol agent (strain PR11), of black pod rot promising but not as effective as Ridomil, under the high disease pressure. According to Adedeji, *et al.*, (2010) *Trichoderma* spp. as bio-agents were successfully combined with fungicides thereby reducing the frequency of fungicide application from four to one with significant pod-rot reduction on the field, comparatively high yield and/or more profit (high revenue-cost-ratio).

2.4.2.1.1 Endophytic fungi

Many researchers have studied the *in vitro* efficiency of endophytic fungal isolates from cocoa against pod rot caused by *Phytophthora*. Tondje *et al.* (2006) reported that, cocoa pod husk pieces pre-treated with endophytic fungus *Geniculosporium* strain BC177 significantly reduced *P. megakarya* sporulation. The percentage of fruits infected with *Phytophthora* was reduced significantly by *C. gloeosporioides*, an endophytic fungus isolated from cocoa (Mejia *et al.*, 2008). When endophytic *Trichoderma martiale* strain ALF 247 was applied at concentrations ranging from $1x10^4$ to $5x10^7$ conidia ml^{-1} resulted in decreased disease severity by *Phytophthora palmivora* in cocoa (Hanada *et al.*,2009). Out of 139 *Trichoderma* isolates, Twenty-five isolates of native *Trichoderma* reduced the mycelial growth of *P. Palmivora* more than 50 per cent. The isolate T17 assigned to *T. virens* reduced mycelium growth upto 97.9 per cent. All isolates except one reduced foliar sensitivity to *P. palmivora*. Twenty-six *Trichoderma* isolates reduced the pod sensitivity to *P. palmivora* more than 50 per cent. (Mpika, *et al.*, 2009).

Arnold *et al.*, (2003) showed the role of endophytic fungi in reducing *Phytophthora* infection, *viz.*, inoculation of endophyte-free leaves with endophytes

isolated from naturally infected, asymptomatic hosts significantly decreased both leaf necrosis and leaf mortality when *T. cacao* seedlings are challenged with a major pathogen (*Phytophthora* sp.). A new species *Trichoderma martiale* was isolated as an endophyte from sapwood in trunks of *Theobroma cacao* (cacao, Malvaceae) in Brazil (Hanada *et al.*, 2008) which was later proved to have a clear-cut potential for the *T. martiale ALF 247* to be used for control of Black-Pod Rot of cacao by Hanada *et al.*, (2009) under field condition.

2.4.2.2 Antagonistic bacteria

Unlike fungal antagonists, studies on bacterial antagonists against *Phytophthora* pod rot of cocoa are relatively scanty. However, Galindo (1992) reported that the epiphytic microflora like *P. fluorescens* and *P. aeruginosa* present on the surface of healthy pods of cocoa were antagonstic to *P. palmivora* and caused reduction in disease incidence under field condition. According to Dennis, *et al.*, (1995), epiphytic bacteria from infected pod surface were antagonistic to *P. palmivora*. Berger *et al.*, (1996) reported that, *Bacillus subtilis* Cot1 controlled *Phytophthora* and *Pythium* damping off in brassica nurseries. Later, Sharifuddin, (2000) also reported potential antagonistic bacteria including *P. aeruginosa* and *Bacillus* sp. against *P. palmivora* and *P. nicotianae*. According to Anith *et al.* (2003), most of the bacterial antagonists screened showed varying levels of antagonism towards *P. capsici* in black pepper in the dual culture and the shoot assay. Epiphytic fluorescent pseudomonads isolated from cocoa pod surface were found effective antagonists against *Phytophthora* (Bhavani, 2004). Zhang *et al.*, (2010) opined that, PGPR strains are effective against *P. capsici* on squash, and improved disease control can be achieved by multiplexing them.

2.4.2.2.1 Endophytic bacteria

Fungal species have been the main focus for research on biological control of cacao diseases, while cacao-associated bacteria have been ignored by nearly all workers. However, application of *B. cereus* isolates BT8 (from tomato) or BP24 (from potato) together with the polysilicon surfactant Silwet L-77 (0.24% vol/vol) resulted in long-

term (>68 days) stable colonization of the bacterium on cacao leaves. Further investigation revealed that foliar colonization by BT8 and BP24 was primarily epiphytic. Significant reductions of disease severity on cacao leaf disks challenged with *P. capsici* were recorded from after 26 days to 68 days following colonization with BT8. (Melnick *et al.*,2008). Aravind *et al.*, (2009) reported three species of endophytic bacteria from roots and leaves of black pepper viz., *Pseudomonas aeruginosa, P. putida* and *Bacillus megaterium* brought about 70 per cent suppression of *P. capsici* infection in black pepper nursery.

2.5 Endophyte - the concept

Mitochondria and plastids of eukaryota may represent the most extreme form of endo-symbiotic relationship by prokaryotes. This is evidenced by their unit membrane, and the physical, genetic and biochemical features that suggest the possibility of their prokaryotic origin. Further, from these structures, a very small circular genophore has been isolated which differs markedly from the host cell (Verma, 1992). This explains the deep rooted relationship between micro organisms and plants.

The idea that non pathogenic bacteria can be present as symbionts in healthy plant tissue without producing obviously detrimental effects has been conceived as early as in 1948 by Sanford. Initially, endophytic bacteria were regarded as latent pathogens or as contaminants from incomplete surface sterilization (Thomas and Graham, 1952). According to Philipson and Blair (1957), the circumstances that cause a passive endophyte to change into pathogen are not known. But they opined that, further studies are needed to establish the probability of an endophyte getting converted into a pathogen. Later studies especially those from 1980s onwards confirmed the ubiquity of endophytes in plants. Multitudes of microbes reside in seeds (Fletcher and Harvey, 1981) leaves (Espinosa-Garcia and Langenheim,1990; Olivares *et al.*,1996), stems (Dong *et al.*, 1994), roots (Narisawa *et al.*, 1998; Varma *et al* .,1999, Cao *et al.*, 2002). Several studies have also suggested that many endophytic associations are not neutral, but are beneficial to plants (Barka *et al.*, 2002; Surette *et al.*, 2003).

The intimate association of bacterial endophytes with plants makes them potential candidates for application in plant protection. Bacteria living inside plant tissues may form associations ranging from pathogenic to symbiotic. In a review by Lodewyckx *et al.* (2002), 81 different bacterial species were reported to form endophytic associations with plants. The beneficial effects that endophytes can confer on plants have made the study of plant-endophyte associations an important research topic for scientists investigating biological control of diseases in annual, biennial, and perennial crops (Bargabus *et al.*, 2004; Kloepper *et al.*, 2004).

2.6 Definitions of an endophyte

Several workers have defined the term endophyte. The earliest was given by Anton de Barry (1866) as microorganisms that colonize internal plant tissues. As this definition included plant pathogens also, Caroll (1986) modified it as asymptomatic microorganisms living inside plants thus excluding pathogens. According to Petrini (1991), endophytes are microorganisms that inhabit, at least for one period or other of their life cycle, inner tissues of plants without causing any apparent harm to the host. As per this definition, many of the organisms important to plant pathology, both deleterious and beneficial, are endophytes.

Wilson (1995) modified the definition by Petrini as bacteria and fungi, that for all or part of their life cycle invade the tissues of living plants and that cause unapparent or asymptomatic infections to the plant host. A more practical definition has been put forward by Hallmann *et al.* (1997), which is accepted by most of the workers according to which, endophytes are microorganisms that can be isolated from surface disinfested plant tissues or extracted from within the plant and do not visibly harm the plant. But later studies showed that not all endophytes could be obtained in culture. So, Azevedo *et al.* (2000) defined them as microorganisms that are culturable or not, that inhabit interior of plant tissues, causing no harm to the host and that do not develop external structures excluding in this way nodulating bacteria and mycorrhizal fungi.

Criteria to recognize "true" endophytic bacteria have been published (Reinhold-Hurek and Hurek 1998) and this requires not only the isolation from surface- disinfected

tissues but also microscopic evidence to visualize "tagged" bacteria inside plant tissues. The latter criterion is not always fulfilled. Use of the term putative endophytes has been recommended for those not validated microscopically. True endophytes may also be recognized by their capacity to reinfect disinfected seedlings (Rosenblueth and Martínez-Romero, 2004).

2.7 Isolation of endophytes

Different methods have been adopted for isolation of endophytic microorganisms *viz.*, homogenization and vacuum methods. Gardner *et al.* (1982) had made the observation that better efficiency of homogenization of both root and twig of citrus xylem compared with vacuum methods. Homogenization or trituration of surface sterilized roots of sugarcane plants treated with five per cent NaOCl for 1 h. yielded endophytic diazotrophic bacteria. However, this treatment was less effective with roots of *maize* (Mc Clung *et al.*, 1983). In the protocol for isolation of endophytes, described by Petrini (1986), the leaf and stem samples were washed twice in distilled water, then surface sterilized by immersion for one minute in 70 per cent (v/v) ethanol, four minutes in sodium hypochlorite (three per cent (v/v) available chlorine) and 30 seconds in 70 per cent (v/v) ethanol and then washed three times in sterilized distilled water for one minute each time. After surface sterilization, the samples were cut into 5-7 mm pieces and aseptically transferred to plates containing potato dextrose agar PDA.

In vacuum extraction (Bell *et al.*, 1995), which used a vacuum extraction apparatus for isolation of endophytes the sap extracted using the apparatus was used for spread plating on suitable medium. Bell *et al.*, (1995) compared the efficiency of vacuum extraction with trituration method for isolation of endophytes. They could get 3.83×10^3 g^{-1} to 1.31×10^4 g^{-1} colony forming units of endophytic bacteria from grape vine by homogenization of xylem tissue, whereas vacuum extraction yielded only 2.65×10^2 ml^{-1} to 3.46×10^3 ml^{-1} colonies from different samples collected. Many bacteria attached to vessel walls were observed by them by SEM, and this was suggested as reason for getting more counts through homogenization.

Hallmann *et al.* (1997) considered trituration technique to be ideal for isolation of endophytes as it allows endophytic bacteria to be selectively isolated from vascular tissue in consistently high numbers. In this, surface sterilized leaf/stem/root bits homogenized into a fine paste with suitable sterile buffer in a sterilized mortar and pestle under aseptic conditions. The homogenate was then serially diluted in the same buffer and plated on appropriate medium. However, trituration technique is the most popular as it yielded maximum endophyte count (Rai *et al.*, 2007)

Yet another method was used by (Hanada *et al.*, 2008) who isolated endophytic fungi directly from the sap wood of cocoa tree. Bark was removed from the tree using a sharp, surface-sterilized knife; and immediately five small pieces of the freshly revealed sapwood, each piece of size $25mm^2$, was removed immediately with a flamed scalpel and placed in a Petri plate containing 20ml potato-dextrose agar (PDA) with 25 mg ml^{-1} chloramphenicol and incubated in darkness at 25°C. Individual fungi were recovered as they grew out of the wood. From cocoa leaves endophytes were isolated by briefly washing in running tap water followed by surface sterilisation in 0.525 per cent sodium hypochlorite for three minutes and 70 per cent ethanol for two minutes then immersed in sterile water for one minutes; and then placed on two per cent malt extract agar (Mejia *et al.*, 2008).

One of the central problems with the isolation and identification of endophytic microbes is the elimination of surface microflora. Sterilizing by chemical means has been the usual strategy used to recover endophytic bacteria that persist intercellularly or intracellularly in plant tissues. However, techniques to surface sterilize tissues are subject to wide variations due to different growing conditions, the age of the plants, and structures which demand experimentally determined surface sterilization methods. Thus, different kinds of surface sterilization methods have been employed for isolation of endophytes from various types of plants (Mc Clung *et al.*, 1983; Jacobs *et al.*, 1985; Fisher *et al.*, 1992; Bell *et al.*, 1995; Mc Inroy and Kloepper, 1995; Shishido *et al.*, 1999). Usually the first step is the surface sterilization followed by transfer of plant segments or the diluted homogenate to appropriate culture medium. According to the

crop, plant part used or research objective, modifications in the basic procedure may be adopted (Araujo *et al*, 2002).

Surface sterilization of sugarcane roots with one per cent Chloramine T has been used to isolate endophytic and diazotrophic *A. diazotrophicus* and *H. seropedicae* bacteria (Baldani *et al.*, 1986; Cavalcante and Dobereiner, 1988). Arnold and his coworkers described the procedure they followed in which, apparently healthy leaf samples were collected, brought in sterile polythene bags to the laboratory and processed within 24 h of collection. Surface sterilization of samples was done by cleaning leaves under running tap water and cutting them into segments of one cm length followed by stepwise washing with 70 per cent ethanol for two min, sodium hypochlorite solution for five min and 70 per cent ethanol for 30 s followed by two rinses in sterile distilled water, then allowed to surface dry under sterile conditions (Arnold *et al.* 2000).

The efficacy of surface sterilization was confirmed by pressing the sterilized leaf segments on to the surface of PDA medium. The absence of growth of any microorganism on the medium confirmed that the surface sterilization procedure was effective (Schulz *et al.* 1993).

2.8 Distribution and diversity

2.8.1 Endophytic bacteria

The diversity and distribution of endophytic bacteria was first observed by Gardner *et al.* (1982), who identified bacteria present in the xylem fluid from the roots of the rough lemon rootstock of the Florida citrus tree. Among the 13 genera found, the most frequently occurring genera were *Pseudomonas* (40per cent) and *Enterobacter* (18per cent), which were regarded as the dominant while the others were classified as rare species. Jacobs *et al.* (1985) have listed the seven most commonly observed bacterial genera associated with apparently healthy sugar beet root tissue. These include *Bacillus subtilis, Erwinia herbicola, Pseudomonas aeruginosa, P. fluorescens, Corynebacterium* sp., *Lactobacillus* sp. and *Xanthomonas* sp. . Similarly, Leifert *et al.* (1989) isolated 190 strains of bacteria from micro propagated plant cultures of which 30 per cent were identified as belonging to the genus *Pseudomonas*. Both Gram-negative and -positive bacteria (including *B. subtilis*) have routinely been isolated from maize and

other plants (Fisher *et al.*, 1992). In most of the studies, bacteria have been isolated from within the crop plants like sugarcane (Dong *et al.*, 1994), sweet corn (Mc Inroy and Kloepper, 1995) *Zea mays* L and *Zea luxurians* (Palus *et al.*, 1996 rice (Barraquio *et al.*, 1997) cotton (Quadt-Hallmann *et al.*, 1997) potato (Sturz *et al.*, 1998) and from *Zea mays* (Chelius and Triplett, 2000). Among the microbes isolated from rhizosphere, phyllosphere, endorrhiza and endosphere of field-grown potato, the most prominent species of all microenvironments was *Pseudomonas putida* and the rhizosphere and endorrhiza were the main reservoirs for antagonistic bacteria (Berg *et al.*, 2005). Yet another report says that, from tissue culture plants of *Castanea sativa,* among the endophytes isolated, *Bacillus* and *Pseudomonas* were the most abundant isolated genera. In rooting phase, 10 isolates were also obtained. (Ferrador *et. al.*, 2005).

Many different bacteria have been isolated from surface-sterilized tissues of monocots and dicots, from crop and non crop plants, and from herbaceous and woody plants. (Bell *et al.*,1995; Hallman *et al.*, 1997; Bent and Chanway, 1998; Shishido *et al.*, 1999). Sturz *et al.* (1998), studied endophytes in crop rotation who found that, red clover (*Trifolium pratense*) and potatoes, share specific associations of bacterial endophytes. Twenty-five bacterial species from 18 genera were common to both clover and potatoes and represented 73 per cent of all the bacteria recovered from clover root tissues and 73 per cent of all the bacteria recovered from potato tubers.

Gagne *et al.* (1989) observed that, out of 33 endophytic strains obtained from alfalfa, only three were pathogenic. This finding was supported by (Bell *et al.*, 1995) who found that, Identification of endophytes collected from grape vine stem revealed predominance of gram negative bacteria and only six strains among them were known to be phytopathogenic.

The population density of endophytic bacteria is highly variable, depending on the species, host genotype, the host developmental stage, inoculum density, and environmental conditions (Pillay and Nowak 1997). In general endophytic bacteria occur at lower population densities than rhizospheric bacteria or bacterial pathogens and

endophytic populations, like rhizospheric populations, are conditioned by biotic and abiotic factors but they could be better protected from biotic and abiotic stresses than rhizospheric bacteria (Hallmann *et al.*, 1997). Interestingly, this is also the case with epiphytes (on leaf surface), which are highly variable in number, varying around 1000-fold the population size of one individual species from leaf to leaf (Mercier and Lindow 2000). Tan *et al.* (2003) also opined that the population of endophytes vary among plants. Rosenblueth and Martínez-Romero (2004) found that, endophytes occur in lesser population compared to rhizosphere bacteria and according to Seghers *et al.* (2004), the endophytic population is affected by atmospheric factors.

The genotypic diversity of indigenous bacterial endophytes within stem of tropical maize (*Zea mays* L.) was determined in field and greenhouse experiments by Rai *et al.* (2007). Endophytes were found in most of the growing season at populations ranging from $1.36–6.12 \times 10^5$ colony-forming units per gram fresh weight (cfu g^{-1} fresh weight) of stem. Analysis of these bacterial endophytes led to the identification of *Bacillus pumilus, B. subtilis, Pseudomonas aeruginosa and P. fluorescens* as the relatively more predominant group of bacterial species residing in maize stem. Taghavi *et al.*, (2009) reported that, members of the Gammaproteobacteria dominated a collection of 78 bacterial endophytes isolated from poplar and willow trees.

Recent studies showed that there was significant difference in endophytic colonization and the type of endophytes between root and leaf tissues. Endophytic bacteria were isolated from both the roots and the stems of sugarcane plants by Mendes *et al.*, (2007) with a significantly higher density in the roots. Similarly, according to Shankar-Naik *et. al.* (2009), the extent of colonization of dominant endophytes and the rate of infection were more in roots (30.23 per cent) than leaves (17.24 per cent). Colonization frequency of some dominant endophytes was also higher in root segments than leaf segments.

2.8.2 Endophytic fungi

Unlike mycorrhizal fungi that colonize plant roots and grow into the rhizosphere, endophytes reside entirely within plant tissues and may grow within roots, stems and/or leaves, emerging to sporulate at plant or host-tissue senescence (Sherwood and Carroll, 1974; Carroll, 1988). Studies of endophytic fungi in woody plants show that they are highly abundant and diverse, particularly in the tropics (Arnold *et al.*, 2000). Since cocoa grows in the wild as a forest under storey tree in many tropical regions of Central and South America (Wood and Lass, 2001) and these forests are some of the most diverse ecosystems in the world, several studies of the endophytic and epiphytic fungi associated with cacao have been carried out (Arnold *et al.*, 2003; Arnold and Herre, 2003; Evans *et al.*, 2003), but still, it is likely that only a small part of the vast microbial diversity associated with cocoa has been described. High diversity, spatial structure, and host affinity among fungal endophytes associated with cocoa across lowland of Panama has been documented by Arnold and coworkers (2003). Endophytic fungi from cocoa with biocontrol potential has been isolated by many researchers (Rubini *et al.*, 2005; Tondje *et al.* ,2006; Mejia *et al.*, 2008; Hanada *et al.*, 2009; Mpika, *et al.*, 2009).

The greater number of fungal isolates in winter and monsoon seasons than in the summer season has been reported by Wilson and Carroll (1994).This suggests that colonization by endophytes is correlated with climatic factors and the fungi isolated in any work are possibly the most easily selected under the culture conditions used. According to Lodge *et al.* (1996), quantitative surveys of endophyte colonization patterns may be sensitive to leaf size, age, methodology, and growth medium. Schulthess and Faeth (1998) found that climatic factors may determine spread and germination success of endophytic fungal spores who suggested that, fungi such as *Colletotrichum, Phomopsis* and *Pestalotiopsis* produce slimy conidia that are not forcibly released but dispersed by water, which may account for increased isolation in the wet season. On the other hand, in winter high humidity and moderate temperature may allow the fungal propagules to germinate successfully. The higher incidence of species in the genera *Cladosporium* and *Penicillium* isolated during the summer may be

due to the ability of their spores to survive and even grow at low water potentials. Gamboa *et al.*, (2002) reported that the predominant endophyte genera found in all tropical plant species surveyed were *Xylaria*, *Colletotrichum*, and *Phomopsis*.

2.8.3 Endophytic yeasts

In addition to filamentous fungi, bacteria and actinomycetes, significant numbers of endophytic yeasts are also reported to be present inside live plant tissues. Endophytic yeasts have been isolated from various plant species including the cordgrass *Spartina alterniflora* (*Pichia spartinae*) (Meyers *et al.*, 1975), tomato leaves (*Rhodotorula* sp.) (Larran *et al.*, 2001), wheat leaves (*R. rubra* and *Cryptococcus* sp.) (Larran *et al.* 2002), banana roots (Cao *et al.*, 2002), tissue cultures of various plants (Bunn and Tan, 2002). *Acrostichum aureum* rhizomes (Maria and Sridhar, 2003), and rice leaves (Tian *et al.* 2004), However, they were not tested for their potential as biocontrol agents or as plant growth promoters. Although endophytic yeasts have been shown to promote maize growth under gnotobiotic and glasshouse conditions (Nassar *et al.* 2005), no attempts to date have been made to use endophytic yeasts to protect plants under actual field condition (El-Tarabily, and Sivasithamparam, 2006).

2.9 Use of molecular techniques for enumeration of endophytes

Endophytic bacteria have been studied mainly after culturing in laboratory media, but a more complete scheme is emerging, *viz.*, using methods that do not require the bacteria to be cultured and that make use of the analysis of sequences from bacterial genes obtained from DNA isolated from inside plant tissues (Engelhard *et al.*, 2000; Sessitsch *et al.*, 2002). It has been hypothesised that, there exist endophytes that have not yet been cultured and the proof for this comes from the study of citrus endophytes by denaturing gradient gel electrophoresis profiles of 16S rRNA gene fragments amplified from total plant DNA in which, some bands did not match any of the isolated bacteria grown in culture media (Araujo *et al.* 2002). This technique has been used by Reiter *et al.* (2003) for studying endophytic population in potato. Miyamoto *et al.* (2004) found novel endophytic nitrogen-fixing clostridia from the grass *Miscanthus sinensis* as

revealed by terminal restriction fragment length polymorphism analysis. Following this molecular approach for studying wheat endophytes in Australia revealed a larger diversity of actinobacteria than that obtained by culturing endophytes (Conn and Franco 2004). Where as in another study by Cankar *et al.*, (2005), no differences were obtained by culturing or culture-independent methods and both revealed similar bacteria from the genera *Pseudomonas* and *Rahnella* in Norway spruce seeds.

2.10 Endophytes as bio control agents

Research on biological control agents has emphasized free living, plant associated nonpathogenic bacteria present in the rhizosphere and phyllophane. These organisms have been successfully used in the management of soil borne diseases. But their success is limited when used in the management of aerial plant parts. This is due to the failure of establishment of bio control agents at target points. The reasons are many like extreme environmental conditions, lack of a sufficient space and media for multiplication on the plant surface, competition from natural epiphytic microflora present *etc.* These difficulties are circumvented by endophytes which reside within the interior regions of plants (Downing and Thomson, 2000). Endophytic microbes have several attributes which made them attractive as potential biocontrol agents. They colonize and form associations within plant tissues without causing disease, are protected from variable environmental conditions and except for other endophytes from competition for limited nutrients and they make use of plant sap as their medium of multiplication (Azevedo *et al.*, 2000).

2.10.1 Endophytic bacteria

Efficiency of endophytic bacteria in the control of wilt diseases like Oak wilt (*Urocystis fragacearum*) by endophytic *Pseudomonas denitrificans and P. putida*) (Brooks *et al.*, 1994) and Fusarium wilt of cotton by *P putida , P, corrugata* and *Bacillus pumilus etc.* (Chen *et al.*, 1995) had been reported. According to Pleban *et al.* (1995); these bacteria move upward and downward from the point of application and by colonizing the internal tissues, exclude the entry of a pathogen in the vascular stele. Thus, endophytic bacteria have brought about significant control of *Fusarium solani* in cotton, *Sclerotium rolfsii* in beans (Pleban *et al.,* 1995) and *Fusarium oxysporum* f. sp.

pisi in peas (Benhamou *et al.*, 1996b). Similar results have also been reported by Krishnamoorhty and Gnanamanickam (1997) and in rice and M'Piga *et al.*, (1997) in pea and tomato.

Endophytic bacteria seem to be able to lessen or prevent the deleterious effects of phytopathogenic organisms, and this has been reported by many workers. Sturz *et.al.*,(1997) tested the endophytic flora from potato and clover which were grown as inter crop for antagonism towards *Rhizoctonia solani* and they found that, of the bacteria tested, 74 per cent showed some degree of *in vitro* antibiosis to the clover and potato pathogen. Further based on the study, it was postulated that, such endophytic intercrop bacterial associations appeared to be complementary in nature and support the view that there are microbial benefits to be gained from clover in crop sequences with potatoes, beyond those of the residual nitrogen left in the soil and the organic matter added. In another study it was found that, endophytic bacterial strains 73a and A1a inhibited mycelial growth of *Verticillium dahliae* strains JC1B and BP2 on cotton (*Gossypium hirsutum)* by 51.0-53.3 per cent respectively (Fu, *et. al.,* 1999). Viswanathan *et al.*, (2003) reported that, endophytic isolates, of *Pseudomonas aeruginosa, Pseudomonas fluorescens* and *Pseudomonas putida* showed *in vitro* antagonism towards the red rot pathogen of sugarcane *Colletotrichum falcatum* and they have also reported reduction in the red rot disease development by application of endophytic strain of *Pseudomonas fluorescens* in sugarcane.

Nejad and Johnson (2000) have found that endophytes from oil seed and rape significantly reduced disease symptoms caused by vascular wilt pathogens *Verticillium dahliae* Kleb and *Fusarium oxysporum* f. sp. *lycopersici* (Sacc.). Benhamou *et al.*,(2000) reported that, treatment with the endophytic bacterium *Serratia plymuthica* strain R1GC4 prior to *Pythium* inoculation resulted in less seedling disease development in cucumber (*Cucumis sativus*) as compared with that in non treated control plants. Reiter *et al.* (2003), isolated endophytic strain of *Clavibacter michiganensis* from potato with biocontrol activities against *Erwinia carotovora*. As opined by Bacon and Hinton (2002) for the management of pathogens with endophytic growth, endophytic biocontrol agents are specially suited and they have reported the endophytic strain of *B. mojavensis*is

which is antagonistic to the fungus *Fusarium moniliforme*, which is also an endophytic mycotoxin-producing pathogen of maize and other plants.

Barka *et al.*, (2002) reported that, endophytic *Pseudomonas* sp. strain PsJN, demonstrated antagonistic effect on *in vitro* growth and development of *B. cinerea*. It was observed that precolonization of banana roots with *Pseudomonas fluorescens* could reduce *Fusarium oxysporum f. sp. cubense* colonization by 72per cent (Mohandas *et al.*, 2004). Sessitsch *et al.*, (2004) found that, seven out of 35 endophytes were able to antagonize fungal as well as bacterial pathogens and showed a high production of active compounds and were therefore considered as promising biological control agents.

Rajendran *et al.*, (2006) has opined that, among biological control methods, endophytic bacteria are an alternative to systemic pesticides that can be more reliable and ecologically as well as economically sustainable. According to Melnick *et al.* (2008), natural cacao endospore-forming bacterial endophytes may be better suited for long-term colonization of cacao and could activate plant defense mechanisms more successfully than introduced fungal bio control agents. They have also suggested that the application of bacteria native to the region could reduce the regulatory and environmental concerns associated with use of non-native microbes. Further they found significant reductions of disease severity on cacao leaf discs challenged with *Phytophthora capsici* were recorded from after day 26, and through 68 days following colonization with *Bacillus cereus* isolates (BT8 from tomato). They have opined that, broad spectrum resistance provided by endophytes would be beneficial in cacao growing regions where trees are often under simultaneous pressure from multiple diseases including witches' broom, frosty pod, and black pod rot. Similarly in grapevine, several endophytic bacteria exhibited dual antifungal mechanism through direct antagonism and induction of plant defense reactions, while most bacteria-induced plant defense reactions only (Trotel-Aziz, 2008). According to Bailey *et al.* (2008), endophytic microbes offer unique candidates for bio control of cocoa diseases. Aravind *et al.*, (2009) reported three species of endophytic bacteria from roots and leaves of black pepper *viz.*, *Pseudomonas aeruginosa, P. putida* and *Bacillus megaterium* which brought about 70 per cent suppression of *P. capsici* infection in black pepper nursery.

2.10.2 Endophytic fungi

Redman *et al.*, (1999) reported a non-pathogenic mutant of *Colletotrichum magna* (path-1) colonized cucurbits and protected watermelon (*Citrullus lanatus*) and cucumber (*Cucumis sativus*) seedlings from anthracnose disease caused by wild-type *C. magna*. In cocoa the role of fungal endophytes as bio-control agents has been proved by many researchers. Arnold *et al.* (2003) found that inoculation of endophyte-free leaves with endophytes isolated frequently from naturally infected, asymptomatic hosts significantly decreased both leaf necrosis and leaf mortality when *T. cacao* seedlings were challenged with a major pathogen (*Phytophthora* sp.). Holmes *et al.* (2004) also showed the ability of *Trichoderma ovalisporum* TK-1 to enter the intact plumule of cocoa seedling and establish itself in the apical meristem and the younger tissues of the stem. But according to them, it still remains to be determined; however, if fungal endophytes can proliferate systemically and persist as permanent residents within cocoa pods, thereby conferring resistance to systemic pathogens such as *Moniliophthora* spp. Tondje *et al.* (2006) reported the efficacy of endophytic fungus *Geniculosporium* strain BC177 against *P. megakarya* sporulation.

Endophytic fungi have been used against oomycete pathogens of other crops also. Kim *et al.*, (2007) reported that, among endophytic fungi isolated from healthy tissues of vegetable plants, *F. oxysporum* EF119 showed the most potent *in vivo* anti-oomycete activity against tomato late blight and *in vitro* antagonism towards several oomycete pathogens. In dual-culture tests, it inhibited the growth of *Pythium ultimum*, *P. infestans* and *Phytophthora capsici*.

Field trials assessing the effects of three endophytic fungi (*Colletotrichum gloeosporioides*, *Clonostachys rosea* and *Botryosphaeria ribis*) on pod loss due to *M. roreri* and *Phytophthora* spp. were conducted by Mejia and associates (2008) in which, treatment with *C. gloeosporioides* significantly decreased pod loss due to these diseases. They observed that, treatment with *C. rosea* (endophyte) reduced the incidence of cacao pods with sporulating lesions of *M. roreri* by 10 per cent. A new species *Trichoderma martiale* was isolated as an endophyte from sapwood in trunks of *Theobroma cacao* (cacao, Malvaceae) in Brazil (Hanada *et al.*, 2008) and they have reported that the

selected *Trichoderma* isolate has good endophytic ability, in addition to other attributes such as mycoparasitism, antibiosis, and/or induced resistance could greatly improve the possibilities of developing functional biocontrol strategies for cacao diseases.

The possibility of endophytes as biological control agents has been studied in other woody species also. As suggested by Ganley *et al.*, (2008) fungal endophytes could provide a useful alternative or ancillary management tool for combating pests and diseases. They observed that, pine seedlings previously inoculated with fungal endophytes lived longer than endophyte-free seedlings and also showed some reduction in white pine blister rust disease severity. Biocontrol of *Rhizoctonia solani* by the endophytic fungus *Cladorrhinum foecundissimum* in cotton plants has been reported by Gasoni and de Gurfinkel, (2009). In another report, it was found that, out of 139 *Trichoderma* isolates, twenty-five isolates of native *Trichoderma* reduced the mycelial growth of *P. Palmivora* more than 50 per cent. One isolate (*T.virens*) reduced mycelium growth upto 97.9 per cent. All isolates except one reduced foliar sensitivity to *P. palmivora*. Twenty-six *Trichoderma* isolates reduced the pod sensitivity to *P. palmivora* more than 50 per cent (Mpika, *et al.*, 2009).

2.11 Mode of action

Cook and Baker (1983) suggested different mechanisms by which the endophytic microbes controlled *Fusarium* wilt of different crops. These mechanisms include production of antifungal compounds, siderophore production, nutrient competition, niche exclusion and induction of systemic resistance. It is possible that, several of these mechanisms play a role in the biological control exhibited by these organisms. According to Backman *et al.* (1997), the effectiveness of endophytes as biological control agents (BCAs) is dependent on many factors. These factors include: host specificity, the population dynamics and pattern of host colonization, the ability to move within host tissues, and the ability to induce systemic resistance. Antibiosis, the production of antimicrobial compounds, and mycoparasitism, the feeding on a fungus by

another organism, are mechanisms whereby *Trichoderma* species provide protection to plants against plant pathogens (Chet *et al.*, 1998; Howell, 2003; Harman *et al.*, 2004).

Production of volatile inhibitory substances by endophytes were studied by (Negad and Johnson, 2000). They found that, most of the endophytic isolates from oil seed rape were HCN negative but the isolates produced volatile metabolites which had fungal inhibitory action. Hence they concluded that the endophytes are producing antifungal volatiles other than HCN. Volatile substances such as 2-3 butanediol and aceotin produced by bacteria have been reported to be responsible for plant-growth promotion (Ryu *et al.*, 2003). It is yet to be determined if volatiles could be produced inside plants.

Microscopic observation of *B. cinerea* mycelium from the zone of contact between the fungus and with *Pseudomonas* sp. on the potato dextrose agar plate by Barka *et al.*, (2002) showed growth disruption of fungal mycelium, coagulation, and leakage of protoplasm. Compared to the knowledge of mechanisms of antagonism of fungal endophytes, relatively little is known about bacterial endophytes. Bacon and Hinton (2002) opined that, biological control strategy utilizing endophytic bacteria is expected to operate under the general mechanism of competitive exclusion, since bacterial growth within the intercellular spaces would preclude or reduce the growth by other microorganisms such as the intercellular hyphae of *F. Moniliforme.* Observations on the inhibitory response suggested that not all strains produced the same inhibitory substance. Some strains caused fungal lysis upon contact with hyphae, which eventually resulted in lysis of the entire fungal colony (contact inhibition). Other bacteria produced a diffusible inhibitory substance into the medium that produced necrotic areas in hyphae along the edge of a colony. They have discussed the variation in potency and the type of antagonism exhibited by different strains of endophytic bacteria reflected the variation in either the amount or the types of inhibitory substances produced, which also might be unstable or poorly diffused into the agar. Alternatively, each strain may have membranes that are differentially permeable to the inhibitor, thereby restricting its diffusion into the medium. Another possibility suggested is that, the fungus is inhibited by non-antibiotic mechanisms. However, the differences in the appearance in the hyphae due to either

contact or diffusional inhibition suggest that there is probably more than one inhibitor produced by strains.

According to Mejia *et al.* (2008), of tested endophytic 52 morphospecies, of endophytic fungi from cocoa, 40per cent(21/52), 65 per cent (28/43) and 27per cent (4/15) showed *in vitro* antagonism against *Moniliophthora roreri* (frosty pod rot), *Phytophthora palmivora* (black pod rot) and *Moniliophthora perniciosa* (witches broom), respectively. The most common antagonistic mechanism was simple competition for substrate. Nonetheless, 13 per cent, 21 per cent, and zero per cent of tested morphospecies showed clear antibiosis against *M. roreri*, *P. palmivora*, and *M. perniciosa*, respectively.

Macı´as-Rubalcava *et al.*, (2008) first reported production of allelochemicals with antifungal activity by the newly discovered endophytic fungus *E. gomezpompae.* They observed the antagonism by the endophyte towards (fungoid oomycetes) *Phytophthora capsici* and *Phytophthora parasitica*, and the fungi *Fusarium oxysporum* and *Alternaria solani.*

2.11.1 Siderophore production

Findings by Cao *et al.* (2005) indicated the potential of siderophore-producing *Streptomyces* endophytes for the biological control of Fusarium wilt disease of banana whereas, among the endophytic bacteria from sunflower none of the strains produced siderophores (Forchetti *et al.*, 2007). A total of 29 endophytic strains were isolated from the halophyte *Prosopis strombulifera* grown under extreme salinity (Sgroy *et al.*,2009), however, only one was able to produce siderophores, and none of them solubilised phosphate. Whereas, all 37 endophytic strains of *Methylobacterium* spp. isolated from citrus were CAS-positive for siderophore production. *Methylobacterium* spp. produced hydroxamate-type, but not catechol-type siderophores. It was observed that, *in vitro* growth of *Xylella fastidiosa* subsp. *pauca* was stimulated by the presence of supernatant siderophores of endophytic *Methylobacterium mesophilicum* (Lacava *et al.*, 2008).

Kajula *et. al.* (2010) reported siderophore production by endophytic fungus. The siderophore produced *in vitro* was ferricrocin, quantities ranging between 7.9 and 17.6

mgl^{-1}. Only the fungi with antibacterial activity produced ferricrocin and any well-known siderophores were not detected in the broths of antioxidant-producing fungi.

2.12 Induced systemic resistance

White and Cole (1985), opined that, endophytes are important in epidemiology because endophytic associations lead to the enhancement of the resistance of the plant. Later, Viswanathan (1999) and Viswanathan and Samiyappan (1999) revealed the ability of endophytic *P. fluorescens* strain EP1 isolated from stalk tissues of sugarcane in inducing systemic resistance against red rot (*Colletotrichum falcatum*). Benhamou *et al.*,(2000) studied the potential of the endophytic bacterium *Serratia plymuthica* strain R1GC4 in stimulating defence reactions in cucumber (*Cucumis sativus*). Histological investigations of root samples revealed striking differences in the extent of plant defence reactions between bacterized and non-bacterized plants. This resulted in restriction of fungal colonization to the outermost root tissues of bacterized seedlings which was correlated with the deposition of enlarged callose-enriched wall appositions at sites of potential pathogen penetration. As a result of greater ISR in plants, growth was enhanced and disease reduced in many crops (Adhikari *et al.*, 2001; Bacon and Hinton, 2002). Inducing plant defence mechanisms has been suggested as an important benefit rendered by endophytic microbes (Bargabus *et al.*, 2002). Accordingly in grapevine, several endophytic bacteria exhibited dual antifungal mechanism through direct antagonism and through inducing the plant defense reactions, while most bacteria-induced plant defense reactions only (Trotel-Aziz, 2008). Melnick *et al.* (2008) have opined that, an understanding of the defense mechanisms in cacao after endophyte colonization would contribute to optimal application of biological control by providing insights into the possibility of broad spectrum disease suppression. This endophyte-mediated resistance was found to be effective over time, indicating persistence, and is hypothesized to be a form of induced resistance (Ganley *et al.*, 2008).

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

plants than in uninfected plants. Glucosides, lipids, and phenolics were detected in the electron-dense aggregates forming the core of wall appositions formed in cucumber roots when treated with endophytic bacterium *Serratia plymuthica* offering protection against *Pythium ultimum* (Benhamou *et al.*, 2000). Barka *et al.*, (2002) have proved that, bacterized grapevines' plantlets are sturdier, with more lignin deposits. Phenolic compounds enhanced the mechanical strength of the host cell walls and also inhibited the invading *Xanthomonas axonopodis* pv. *malvacearum* (Rajendran *et al.*, 2006). Another report have also stated a relatively higher quantity of lignification (30 - 100% over control) in the bacterized roots compared to the plants untreated which resulted in significant root rot suppression (Ganley *et al.*, 2008).

Other than these defence related compounds, increase in the level of defence related enzymes has also been reported by various workers as a result of treatment with endophytes. The chitinases and β-1, 3 glucanases (which are classified under the PR-3 and PR-2 groups of PR proteins respectively) are reported to be associated with greater resistance in plants induced by endophytes against pests and diseases (Maurhofer *et al.*, 1994; van Loon, 1997). Higher levels of PO have been correlated with enhanced ISR in several plants treated with endophytes (Kandan *et al.*, 2002; Ramamoorthy *et al.*, 2002). Barka *et al.*, (2002) have proved that increased levels of peroxidase (PO), catalase, phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO) were induced in leaves apart from the roots of treated plants indicating the systemic protection offered to black pepper by the strains exploring the prevention of even foliar infection by the pathogen, *Phytophthora capsici*. Rajendran *et al.* (2006) has also reported cotton plants treated with endophytic bacteria and challenged with *X. axonopodis* pv. *malnacearum* (Xam) showed higher levels of PO, as well as PPO.

According to Fu, *et al.*, (1999) endophytic in culture bacteria were able to produce proteins outside the cell but at low concentrations. In the study undertaken by Rajendran *et al.* (2006) timely increase in levels of defense related proteins by pretreatment with PGPE strains prevented infection with *Xanthomonas axonopodis* pv. *malvacearum* (Xam) in cotton under greenhouse conditions.

However in contrast to the aforementioned reports, Arnold *et al.* (2003) found that, instead of inducing systemic defence, the protection offered by endophytic fungal inoculation was primarily localized to endophyte-infected tissues. Further, endophyte-mediated protection was greater in mature leaves, which bear less intrinsic defence against fungal pathogens than do young leaves.

2.13 Plant growth promotion by endophytes

Endophytic bacteria are found in numerous plant species with most of them being members of common soil bacterial genera such as *Pseudomonas, Bacillus, Azospirillum* (Chanway, 1996) and many strains can promote plant growth (Chanway, 1996; Hallmann *et al.*, 1997). Sturz *et. al.* (1998) stated that, out of the 25 isolates of endophytic bacteria tested in potato plant bioassays were predominantly plant growth neutral (56 per cent). The remainders were either plant growth promoting (21 per cent) or plant growth inhibiting (24 per cent). Of the plant growth promoting bacteria, 63 per cent increased shoot height, 66 per cent increased shoot fresh weight, and 55 per cent increased root fresh weight. The effects of plant growth inhibiting bacteria were restricted to reductions in plant height (86 per cent) and shoot fresh weight (36 per cent); root weight was not affected. According to, Fu, *et. al.* (1999), when one isolate of endophytic bacteria (Strain 73a) promoted shoot growth (measured as mean shoot length) by 19.15 per cent, another one (A1a) had no effect on it. Similar observations were made by Nejad and Johnson (2000) who reported that all the endophytic isolates from oilseed rape caused significant increase in shoot fresh weight compared to control. They could also find that endophytes significantly improved seed germination, seedling height and plant growth when used as seed treatment. An average increase of 70 percent over control was recorded in root and shoot growth of corn and beans by Bacon and Hinton (2002) by application of an endophytic strain of *B. mojavensis*.

Although the interaction between endophytes and host plants has not been fully understood, many fungal and bacterial endophytes are reported to promote plant growth and the mechanism attributed includes nitrogen fixation, production of growth-promoting substances and increased resistance to pathogens and parasites (Muthukumarasamy *et al.*, 2002). In a study, conducted by Barka *et al.*, (2002)

grapevine plantlets co-cultured with PsJN (endophytic) bacterium grew faster and had significantly more secondary roots and root and leaf hairs. The response of plant towards bacterization was maintained and amplified after the second generation. Indeed, the bacterium is capable of establishing endophytic and epiphytic populations, allowing clonal multiplication of plantlets by nodal explants *in perpetuum* without the need for reinoculation. In addition, the bacterization induced an enhancement of the fresh weight of shoot and roots as well as the number of nodes per shoot. Bacterized grape vines also had a greater fresh weight of the shoots and roots and faster growth with more lignin deposits (Barka *et al.*, 2002).

In another study, *Bacillus subtilis* strain BS-2 was able to colonize, propagate and move in cabbage plants after inoculation to the plants by seed dipping, watering or leaf daubing. Cabbage plants from seeds dipped in BS-2 suspension for 24 h before sowing recorded a 91.20-138.04 per cent increase in fresh weight compared to the control (Hong, *et al.*, 2004). According to Ryan *et al.* (2008), endophytic bacteria colonize the internal tissues of their host plant and can form a range of different relationships including symbiotic, mutualistic, commensalistic and trophobiotic.They improved the biomass production and the carbon sequestration potential of poplar trees (*Populus* spp.) when grown in marginal soil (Taghavi *et al.*, 2009)

In cocoa no significant difference in growth rate of seedlings was observed by inoculation with the endophytic fungus *Trichoderma stromaticum* (de Souza *et al.*, 2008). Whereas Bae *et al.* (2009), has reported that endophytic colonization of cocoa seedlings by *Trichoderma hamatum* isolate DIS 219b resulted in an increase in the root fresh weight, root dry weight, total fresh weight, root water weight, and total water weight, along with an increase in the root dry weight/ top dry weight ratio leading to delay in onset of drought response. Poplar cuttings *(Populus deltoides _ Populus nigra* DN-34) inoculated with endophytic *Enterobacter* sp. strain 638 repeatedly showed the highest increase in biomass production compared to cuttings of noninoculated control plants (Taghavi *et al.*, 2009).

Plant growth promotion effected by 20 enodphytic isolates from strawberry was evaluated under greenhouse conditions by Dias *et al.*, (2009). The study revealed the

ability of the strains to enhance the root number, length and dry weight and also the leaf number, petiole length and dry weight of the aerial portion. Even under conditions of water stress, endophytic bacteria enhanced growth of sunflower seedlings (Forchetti *et al.*, 2010).

2.13.1 Mechanism involved in growth promotion

Although the interaction between endophytes and host plants has not been fully understood, many fungal and bacterial endophytes are reported to promote plant growth and the mechanism attributed includes nitrogen fixation, production of growth-promoting substances and increased resistance to pathogens and parasites (Muthukumarasamy *et al.*, 2002). The elucidation of the mechanisms promoting plant growth will help to select species and conditions that lead to greater plant benefits.

Several authors have reported various mechanisms by which endophytes exhibit growth stimulation in hosts. Growth promotion as a consequence of nitrogen fixation has been reported by many researchers. Under optimal conditions, some plant genotypes seem to obtain part of their N requirements from nitrogen fixation. These estimates vary widely in different reports and range from 30 upto 80 kg N/ha/year (Boddey *et al.* 1995). Similar observations has been made by Lee *et al.*, (2000) also. It has been reported that N fixation by endophytes increased maize yield in the field (Riggs *et al.*, 2001). Many other workers also suggested N fixation as involved in growth promotion by endophytes (Sevilla *et al.* 2001, Hurek *et al.* 2002). Similarly, nitrogen fixing endophytes seem to relieve N deficiencies of sweet potato (*Ipomoea batatas*) in N-poor soils (Reiter *et al.* 2003). Nevertheless, there is controversy on the level of N fixed by endophytes and the proportion contributed to the plant (Giller and Merckx 2003). Grasses growing in nutrient-poor sand dunes contain members of genera *Pseudomonas*, *Stenotrophomonas* as well as *Burkholderia*. It seems that the *Burkholderia* endophytes could contribute N to the grasses, because nitrogenase was detected with antibodies in roots within plant cell walls of stems and rhizomes (Dalton *et al.* 2004). Inside wheat, *Klebsiella* sp. strain Kp342 fixes N (Iniguez *et al.* 2004). Out of 29 endophytic strains isolated from the halophyte *Prosopis strombulifera* grown under extreme salinity (Sgroy *et al.*,2009), all bacteria were able to grow and to produce some phytohormone such as indole-3-acetic

acid, zeatin, gibberellic acid and abscisic acid in chemically defined medium with or without addition of a nitrogen source.

However N fixation is not the only mechanism by which endophytes improve plant growth. Other mechanisms include production of phytohormones, biocontrol of phytopathogens in the root zone (through production of antifungal or antibacterial agents, siderophore production, nutrient competition and induction of systemic acquired host resistance, or immunity or by enhancing availability of minerals (Sturz *et al.* 2000; Sessitsch *et al.* 2002). Endophytic bacteria possess the capacity to solubilise phosphates, and it is suggested by Kuklinsky-Sobral *et al* (2004) that the endophytic bacteria from soybean participate in phosphate assimilation (Kuklinsky-Sobral *et al.* 2004). A plant-growth-promoting isolate of the yeast *Williopsis saturnus* endophytic in maize roots was found to be capable of producing indole-3-acetic acid (IAA) and indole-3-pyruvic acid (IPYA) *in vitro* in a chemically defined medium (Nassar *et al.*, 2005). Endophytic bacteria from sunflower also expressed P solubilising ability (Forchetti *et al.*, 2007). Endophytic bacteria were isolated from both the roots and the stems of sugarcane plants by Mendes *et al.*, (2007) with a significantly higher density in the roots. Many of them were shown to produce IAA and this trait was more frequently found among bacteria from the stem. Most of the isolates from soybean were also IAA producers (Hung, *et al.*, 2007). Plant growth promotion effected by 20 endophytic isolates from strawberry was evaluated under greenhouse conditions by Dias *et al.*, (2009). The study revealed the ability of the strains to enhance the root number, length and dry weight and also the leaf number, petiole length and dry weight of the aerial portion. The plant growth promotion showed to be correlated to IAA production and phosphate solubilization. Several endophytic bacteria from the halophyte *Prosopis strombulifera* were able to grow and to produce some phytohormone such as indole-3-acetic acid, zeatin, gibberellic acid and abscisic acid in chemically defined medium with or without addition of a nitrogen source (Sgroy *et al.*, 2009). Poplar cuttings (*Populus deltoides* _ *Populus nigra* DN-34) inoculated with *Enterobacter* sp. strain 638 repeatedly showed the highest increase in biomass production compared to cuttings of non-inoculated control plants. Sequence

data combined with the analysis of their metabolic properties resulted in the identification of many putative mechanisms, including carbon source utilization, that help these endophytes to thrive within a plant environment and to potentially affect the growth and development of their plant hosts (Taghavi *et al.*,2009)

2.14 Mode of entry

Endophytic microflora resides within the living tissues of plants and they include fungi, bacteria and actinomycetes. According to Dong *et al.*, (1994) they originate from vegetative planting material. But, more recent works suggests that, most endophytes appear to originate from the rhizosphere or phyllosphere; however, some may be transmitted through the seed (Ryan *et al*, 2007). Successful endophytic colonization goes through several important stages including host finding, recognition, colonization of the plant surface and entrance into internal plant tissue. Pre-colonization interactions include bacterial movement towards root, attachment to plant surface, plant bacterial recognition process at the surface finally penetration through natural openings. Post colonization involves bacterial multiplication and localization within the plant tissue. Endophytic bacteria probably find their host by chemotaxis, accidental encounter or combination of both.

Endophytes may enter through macerated epidermal tissues by pathogen also. Mahaffee and Kloepper (1997) reported increased endophytic bacterial populations as a consequence of *Rhizoctonia solani* infection of *Phaseolus vulgaris*. Nematode infestation also serves as a source of entry for endophytic bacteria (Hallmann *et al.*, 1997). Once entered into the plant system endophytes have to multiply and colonize the plant tissue to establish a successful plant endophytic association.

2.15 Colonization

It has been proved that, bacteria can live within the plants without causing disease by the colonization of legume roots by rhizobia. Other saprophytic bacteria may also be capable of colonizing plant tissue just as bacteria colonizing mammalian tissue (Jacobs *et al.*, 1985). Hence, many researchers have studied the mode of entry of

endophytic bacteria into the plants. Radio labeling has been used successfully to detect the entry and movement of endophytes by Pleban *et al.* (1995). According to Benhamou *et al.*, (1996a), endophytic bacteria move up and down in the conductive tissues of the plant. Auto fluorescent protein (AFP) technique was utilized to detect and enumerate endo/ epiphytic microorganisms and to study the courts of entry to plants (Gage *et al.*, 1996; Tombolini *et al.*, 1997). One of these AFP strategies uses a marker system, which encodes the green fluorescent protein (GFP). It is used for monitoring pseudomonads in root tissues (Tombolini *et al.*, 1997). GFP is a useful AFP biomarker because it does not require any substrate or cofactor in order to fluoresce (Xi *et al.*, 1999). Auto fluorescent protein (AFP) methods are also used widely for studying processes such as microbe–plant interactions and bio-film formation. *(Larrainzar et al., 2005).* Bacterial cells with chromosomal integration of GFP can be identified by epifluorescence microscopy or confocal laser scanning microscopy (Villacieros *et al.*, 2003; Germaine *et al.*, 2004). According to Rosenblueth and Martínez-Romero (2006), true endophytes may be recognized by their capacity to reinfect disinfected seedlings, it is stated that, microscopic evidence to visualize "tagged" bacteria inside plant tissues is essential for considering a bacteria to be an endophyte which is not always fulfilled.

2.16 Methods of inoculation

The methods by which endophytes are applied are similar to those used for applying other biological control agents. Successful colonization and maintenance of effective level of population of endophytic bacteria in the plant and in turn the level of disease control are mainly dependent on the method of delivery of these organisms into the host. Use of endophytes in biocontrol requires introduction of endophytes into plant tissues in the quantity, site and life-history stages that effectively antagonize pathogens (Reinhold-Hurek and Hurek 1998). In the case of grasses, inoculation has been achieved by placing mycelia in coleoptile tissue (Latch and Christensen 1985), syringe inoculation and soaking seeds in spore suspensions (Leuchtmann and Clay 1988). When the endophytic bacteria are introduced into the vegetatively propagated seed, the bacteria survives and moves in the vegetative part and subsequently the propagative seed will

also have the introduced bacteria, thus minimizing the need for frequent application of bacterial strains (Ramamoorthy *et al.*,2001). Sprays of spore suspensions have been used to introduce endophytes into beans and barley (Boyle *et al.*, 2001). Introduction of endophytic bacteria into the host plant at an early growth stage avoids competition for colonization by other micro organisms, and allows attaining required levels of colonization and thus avoiding need of subsequent inoculations (Manjula *et al.*, 2002).

Manjula *et al.* (2002) compared the methods of inoculation of endophytes such as stab inoculation on stems, soaking seeds in bacterial suspensions, methyl cellulose seed coating, foliar spray application of bacteria impregnated granules in furrow, vacuum infiltration and pruned root dip, for introduction of various endopohytes into cotton. They found that no single method of delivery was equally effective for various endophytes such as *Bacillus, Burkholderia, Cellulomonas, Clavibacter, Enterobacter, Phyllobacterium* and *Pseudomonas* spp. into cotton. Hence it was suggested that different methods of inoculation are needed for efficient delivery of diverse strains. Bhowmik *et al.*,(2002) reported that, seed baterization with endophytic *Pseudomonas* sp. was the most effective in reducing cotyledonary infection by Xam. Holmes *et al.*, (2002) successfully introduced endophytes by immersing cacao bud wood in spore suspensions. In another study, *Bacillus subtilis* strain BS-2 was able to colonize, propagate and move in cabbage plants after inoculation to the plants by seed dipping, watering or leaf daubing (Hong, *et al.*, 2004).

Several teams are working on colonization of cocoa by fungal endophytes isolated from different tissues as well as the most conducive inoculation method (Bailey *et al.*, 2008; de Souza *et al.*, 2008; Mejía *et al.*, 2008). However, the establishment of these fungal endophytes in pods requires a systematic and focused effort. According to Mejia *et al.*, (2008), complete descriptions of inoculation methods for endophytes associated with trees are scarce. They used hand-held compression sprayers to apply spore suspensions of endophytes to developing and mature cocoa pods.

2.17 Molecular characterization

Molecular characterization techniques are employed chiefly to study the diversity of the endophytic community and to identify the organism at the species level. The molecular techniques used are 16S rDNA / RNA homology analysis. It should be noted that attempts to evaluate total populations of bacteria in plants may produce varied results, depending on the growth media used for isolation, variations in the growth conditions of the host plant, and the manner in which the plant tissue was used. This is also the case for plant-associated rhizosphere bacterial populations (Kloepper and Beauchamp, 1992). Molecular approaches, based on amplification of the 16S rDNA may be employed to overcome the limitations of classic isolation procedures that are dependent on the isolation of bacteria. The diversity of abundant and metabolically active pseudomonads in potato plants was analysed using the culture independent approach by Reiter *et al.*, (2003). Cloning and sequencing of partial 16S rDNA genes was performed using DNA and RNA extracted from potato stem tissue. Sequence analysis revealed high species diversity, with the most prominent ones being *Pseudomonas stutzeri* and *Pseudomonas gingeri*. Some species showed high rRNA contents indicating high metabolic activity (Reiter *et al.*, 2003). The endophytic bacterial diversity in the roots of rice (*Oryza sativa* L.) growing in the agricultural experimental station in Hebei province, China was analyzed by 16S rDNA cloning, amplified ribosomal DNA restriction analysis (ARDRA), and sequence homology comparison (Sun *et al.*,2008). Among 192 positive clones in the 16S rDNA library of endophytes, 52 OTUs (Operational Taxonomic Units) were identified based on the similarity of the ARDRA banding profiles. Sequence analysis revealed diverse phyla of bacteria in the 16S rDNA library, which consisted of alpha, beta, gamma, delta, and epsilon subclasses of the Proteobacteria, Cytophaga/Flexibacter/ Bacteroides (CFB) phylum, low G+C gram-positive bacteria, Deinococcus-Thermus, Acidobacteria, and archaea (Sun *et al.*, 2008). Trivedi *et al.*, (2010) reported that, 16S rRNA gene clone library analysis of citrus roots revealed shifts in microbial diversity in response to pathogen infection. The clone library of the uninfected root samples has a majority of phylotypes showing

similarity to well-known plant growth-promoting bacteria, including *Caulobacter*, *Burkholderia*, *Lysobacter*, *Pantoea*, *Pseudomonas*, *Stenotrophomonas*, *Bacillus*, and *Paenibacillus*.

Species level identification of endophytes were possible by the use of molecular techniques. The identity of a patented endophytic bacterium was established by 16S rRNA sequence analysis as a strain of *Bacillus mojavensis*, a recently erected species within one of the *B. subtilis* subgroups (Bacon and Hinton, 1999). Sakiyama *et al.* (2001) reported that, many endophytic bacteria were isolated from surface-sterilized coffee cherries. One of the pectinolytic strains was physiologically and phenotypically characterized, and was tentatively identified by partial 16SrDNA sequencing as *Paenibacillus amylolyticus*. A diazotrophic isolate *Pantoea* sp. MY1 and non diazotrophic isolate *Enterobacter* sp. MY2 were identified to the species level by full sequence analysis of 16S rRNA gene. The results showed that MY1 had 99+/-2 per cent similarity to *Pantoea agglomerans* ATCC 27155 and MY2 had 99+/-5 per cent similarity to *Enterobacter asburiae* ATCC 35953 (Asis and Adachi, 2003). The presence of endophytes in Norwey spruce seeds was detected by culturing methods and by direct amplification of the eubacterial 16S rDNA gene. Both approaches identified bacteria from genera *Pseudomonas and Rahnella*. Hence they suggested that plant seeds could serve as a vector for transmission of beneficial bacteria (Cankar *et al.*,2005). Further, a non-pigmented, motile, Gram-negative bacterium designated MTCC 4195T was isolated from surface-sterilized seeds and plant tissue from deep-water rice (*Oryza sativa*) cultivated in Suraha Tal Lake in northern India. This isolate was shown to reinfect and colonize deep-water rice endophytically. Molecular characterization revealed that, the highest level of 16S rRNA sequence similarity (96+/-8 per cent) to strain MTCC 4195T was shown by *Ochrobactrum gallinifaecis* DSM 15295T (Tripathi *et al.*, 2006).

2.18 Genetic engineering of endophytes

Genetic modification of endophytes is considered as a near future application of endophytes in agriculture and medicine. Turner *et al.*, (1991) reported that genetically

engineered endophytic bacterium *Clanibacter xyli* subsp. *cynodontis* isolated from Bermuda grass, was modified to produce an endotoxin from *Bacillus thuringiensis* subsp *kurstaki* for the control of the Europian corn borer in corn. The gene coding for major chitinase of *Serratia marcescens*, ChiA was cloned under the control of tac promoter into the broad-host-range plasmid pKT240 and the integration vector pJFF350 and introduced into *Pseudomonas fluorescens*. It was used as an effective bio control agent for phytopathogenic fungus *Rhizoctonia solani* on bean seedlings (Downing and Thomson, 2000). As reported by Siciliano *et al.* (2001), bacteria degrading recalcitrant compounds are more abundant among endophytic populations than in the rhizosphere of plants in contaminated sites which could mean that endophytes have a role in metabolizing these substances. Accordingly, engineered endophytic *Burkholderia cepacia* strains improved phytoremediation and promoted plant tolerance to toluene (Barac *et al.* 2004).

Materials and Methods

3. MATERIALS AND METHODS

Investigations on "Endophytic microorganism mediated systemic resistance in cocoa against *Phytophthora palmivora* (Butler) Butler" were conducted at the Department of Plant Pathology, College of Horticulture and cocoa farm of Cadbury-KAU Co-Operative Cocoa Research Project (CCRP) attached to College of Horticulture, during 2005-2010.

3.1 ISOLATION OF THE PATHOGEN

The pathogen causing pod rot of cocoa was isolated from infected cocoa pods collected from CCRP cocoa farm on carrot agar medium (CA) (Appendix 1). The pods and leaves were washed under tap water and small bits were cut from infected areas along with healthy portion. These bits were surface sterilized using one per cent sodium hypochlorite solution for one minute followed by washing in three changes of sterile water. The surface sterilized bits were placed on mediated Petri dishes and incubated at 28+/-2°C. When mycelial growth was visible, small bits of the growth were transferred to potato dextrose agar (PDA) slants (Appendix I). The cultural and morphological characters of the isolate *viz.*, rate and pattern of growth, width of hyphae, length of sporangiophores, pedicel length and L/B ratio were studied.

Pathogenicity of the isolate was proved by artificial inoculation on healthy, half matured cocoa pods. Mycelial disc (10mm) of the isolate from seven day old culture grown on carrot agar was placed in a hole of same size made on the pods with sterile cork borer. Cotton moistened with sterile water was placed over the mycelial disc. The inoculated pods were incubated in moist chamber. Observations on the symptom developed were recorded. The organism was re-isolated from pods which were infected by artificial inoculation. The cultural and morphological characters of the isolate were studied and compared with that of the original one. The isolate was purified by hyphal tip method and maintained on PDA slants for further studies.

3.2 ISOLATION AND ENUMERATION OF ENDOPHYTIC MICROFLORA

Endophytic microbes were isolated from samples of feeder roots, tender shoots, leaves and the pods of healthy cocoa plants. Further, samples were collected from cocoa plants grown at three locations from each of the major cocoa growing areas of Kerala *viz.*, Kottayam, Idukki, Pathanamthitta, Thrissur and Palakkad. Fresh samples brought in separate polythene covers were washed under tap water, stored in refrigerator and used for isolation within 48 h. after collection.

3.2.1 Surface sterilization

Since the surface of cocoa plants harbour lot of epiphytes (Galindo, 1992), thorough surface sterilization was needed for eliminating them. The concentration of the sterilant and the time of exposure to it were standardized so as to get the maximum number of endophytes with no growth on sterility check. Three different concentrations of sodium hypochlorite *viz.*, one, two and three per cent were tried for three different exposure time *viz.*, two, five and ten minutes. Further, three different weights of sample of roots, shoots and leaves *viz.*, 250, 500 and 1000 mg were also tried. Since isolation from 1000 mg of sample after surface sterilization with two per cent sodium hypochlorite for 10 min yielded good number of colonies with no growth in the sterility check, it was selected for further studies.

Half matured pods collected were washed and disinfested with 70 per cent ethanol. It was then cut open aseptically using sterile scalpel. A small piece (1 cm^3 approximately) of placenta of the pod was taken and placed in a pre weighed, sterile Petri dish. Weight of the Petri dish along with the piece of tissue was found out. The weight of the tissue was thus calculated and used to find out the number of colonies per gram.

3.2.2 Isolation and enumeration of endophytes

For isolation of endophytes from feeder roots, tender shoots or leaves, the 1g samples were exposed to the sterilant as described above (3.2.1), followed by washing with three changes of sterile water. The root/shoot/leaf bits were then put in sterilized

mortar containing 10 ml sterile potassium phosphate buffer (PB) (0.1 M, pH 7).The bits were finally washed in the buffer. From the final buffer wash, one ml was pipetted out and poured into sterile Petri plate. To this, molten and cooled medium was added and this served as the sterility check. If microbial growth was observed in the sterility check within four days, the isolates obtained from that particular sample were discarded. The surface sterilized bits of root/shoot/leaf/placenta of pod were triturated (Mc Inroy and Kloepper, 1995) using sterile mortar and pestle with 9 ml. of sterile buffer. The triturate was serially diluted in sterile PB up to 10^{-7}. The dilution and medium used for enumeration of each group of micro-organism are furnished in (Table 3.1). One ml of the diluted triturate was pipetted into sterile Petri plate and suitable medium was poured. The plates were incubated at 28+/-2°C for 24h, 48h or seven days as shown in Table 3.1.

Table 3.1 Details of dilution and media used for isolation and enumeration of endophytic microflora

Sl. No.	Organism	Dilution	Medium*	Period of incubation
1.	Fungi	10^{-4}	Martin's Rosebengal streptomycin agar	48h.
2.	Yeast	10^{-4}	Glucose Yeast Extract Peptone Agar	48h.
3.	Actinomycetes	10^{-5}	Ken Knight's Agar	Seven days
4.	Bacteria	10^{-7}	Nutrient Agar	24h.
5.	Fluorescent pseudomonads	10^{-7}	King's B Agar	48h.

* Composition of media given in Appendix I

Representative colonies of endophytes based on colony morphology were picked up from the dilution plates and transferred to respective slants to establish pure cultures following standard protocols. Altogether, 325 endophytic isolates were thus subcultured and their details such as; source (part of the plant from which isolated and place of collection of sample), and medium of isolation were recorded.

3.3 *IN VITRO* ANTAGONISTIC EFFECT OF ENDOPHYTES AGAINST THE PATHOGEN

The *in vitro* antagonistic effect of endophytes towards the pathogen was tested by dual culture method. Initially, 325 endophytic isolates obtained from experiment 3.2.4 were subjected to preliminary screening to test their interaction with the pathogen.

3.3.1 Preliminary screening of endophytic microflora against the pathogen

For preliminary screening, mycelial disc of the pathogen taken from a seven day old culture on PDA was inoculated on fresh PDA plate and incubated for 48h. For screening endophytic bacteria and yeasts, four isolates were inoculated one each on the four sides of the pathogen as a line of streak one cm away from the edge of the Petri dish. The isolates which showed antagonism were selected and transferred to fresh medium. In the case of endophytic fungi, mycelial disc of four isolates were placed one each on four sides of the pathogen one cm away from the edge of the Petri dish. Plates with the pathogen alone served as control. The plates were incubated at room temperature and observed for inhibition of the pathogen for five days or when there was full growth in the control. Based on the preliminary screening, 82 endophytes including 28 isolates of bacteria, 29 isolates of fluorescent pseudomonads, 21 isolates of yeasts and four isolates of fungi which showed antagonism were selected and transferred to fresh medium and pure cultures were established. These antagonistic endophytes were maintained on test tube slants with suitable media by sub culturing at fortnightly intervals. Bacterial isolates were stored in sterile water at 4°C also.

3.3.2 *In vitro* evaluation of antagonistic endophytes

The antagonistic endophytes selected based on the preliminary screening were further tested individually. The objective of this experiment was to select more efficient antagonists from among the 82 endophytes which showed antagonistic action in the preliminary screening.

3.3.2.1 *In vitro* evaluation of antagonistic bacteria and yeasts

All the 57 isolates of bacterial endophytes and 21 isolates of yeasts were evaluated for their antagonistic effect by dual culture method (Utkhede and Rahe, 1983). Mycelial disc (10mm) taken from seven day old culture of the pathogen grown on PDA was placed at the centre of mediated (PDA) Petri dish and incubated for two days. The endophytic bacteria and yeasts were inoculated as a line of streak on both sides, one cm away from the edge of the Petri dish. For each isolate three replications were maintained. Plates with the pathogen alone served as control. The plates were incubated at room temperature, and growth of the pathogen was observed daily, until the control exhibited full growth. The per cent inhibition of the pathogen was calculated using the formula suggested by Vincent (1927).

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

PI = per cent inhibition

C = growth of pathogen in control (mm)

T = growth of pathogen in dual culture (mm)

3.3.2.2 *In vitro* evaluation of antagonistic fungi

Four fungal isolates were evaluated for their antagonistic action against the pathogen by dual culture method (Skidmore and Dickinson, 1976). The organisms were inoculated on dual cultures after giving due consideration of their growth rate. Mycelial disc (10mm) of pathogen from seven day old culture grown on PDA was placed on one side of the plate and incubated at room temperature for two days. Then mycelial disc, (10mm) of antagonistic fungi were placed on the other side of the plate, four cm away from the pathogen and incubated. Three replications were maintained for each isolate. The pathogen grown on monoculture served as control. The plates were observed daily after 24h of inoculation of antagonist till the pathogen grew and covered the plate in control. The per cent inhibition was calculated as in the case of 3.3.2.1.

3.3.3 Selection of antagonistic endophytes

Based on the dual culture screening, 44 isolates which showed more than 40 per cent inhibition of the pathogen were selected for testing their effect against the pathogen on detached cocoa pods and leaves.

3.4 EFFECT OF THE ANTAGONISTIC ENDOPHYTIC ISOLATES IN REDUCING DISEASE ON DETACHED PODS AND LEAVES.

Forty-four selected antagonistic endophytes which consisted of 18 isolates of bacteria 22 isolates of fluorescent pseudomonads and four of fungi were subjected to screening on detached pods to understand their antagonistic efficacy against the pathogen on cocoa pods.

3.4.1.1 Effect of antagonistic endophytes against the pathogen on detached pods.

Healthy, half matured cocoa pods of uniform size were collected from a single clone of cocoa (G VI 10 of CCRP farm), washed thoroughly, and disinfected with 70 per cent alcohol. Spore or bacterial suspension (10^7cfu ml^{-1}) of the antagonistic endophytes was prepared from culture grown on PDA/NA plates. It was sprayed uniformly on the pods and allowed to air dry. The inoculation of the pod with the pathogen was done with and without injury. For this, the endophyte treated pods were marked into two equal halves. On the basal part, inoculation was made after giving injury with pin pricks. Mycelial disc (10 mm) of the pathogen from seven day old culture grown on PDA was placed over the pin pricks and cotton moistened with sterile water was placed over it. Inoculation was made at two sites exactly on the opposite side of each other. On the terminal portion of the same pod, inoculation was made at two sites without giving pinpricks. The inoculated pods were incubated in moist chamber. Pods inoculated with pathogen alone served as control. The procedure was repeated using pods without injury. Observations on length and breadth of the lesions developed were recorded at 48 h interval till pods in control were completely covered by the lesion. The per cent pod area infection was calculated using the formula

$$\text{Per cent pod area infection} = \frac{\text{Length x breadth of lesion}}{\text{Length x breadth of the pod}} \times 100$$

3.4.1.2 Selection of efficient antagonists

Of the selected 44 antagonists screened on detached pods, 25 efficient antagonistic isolates *viz.*, isolates of nine bacteria, 12 fluorescent pseudomonads, and four fungi were selected as they expressed more than 55 per cent effect in reducing infection on cocoa pods.

3.4.2 Effect of antagonistic endophytes against the pathogen on detached leaves

Modified method of detached leaf disc assays originally developed to aid in screening of cocoa genotypes to *Phytophthora* spp. and that have demonstrated to correlate to field resistance (Tahi *et al.*, 2000, Tahi *et al.*, 2006) was used in this experiment. Immature green leaves, collected from a single clone of cocoa were used to study the effectiveness of selected antagonists against the pathogen on detached leaves. For this, 10 to 15cm long, flexuous, and semi-translucent leaves (Bailey *et al.*,2005) were used. The leaves were washed with sterile water and disinfested with 70 per cent ethyl alcohol. Spore/bacterial suspension (10^7cfu ml^{-1}) of endophytes were applied as spray on the leaves and allowed to air-dry. The leaves were placed with adaxial side up on moist sterile filter paper discs placed in sterile Petri dishes (20cm). Pinpricks were given at the centre of the mid rib using sterilized needle. Culture disc (10mm) of pathogen taken from seven day old culture grown on PDA was placed over the pinpricks. Cotton moistened with sterile water was placed over it. The dishes were covered with lid laden with pieces of moist cotton. Leaves inoculated with pathogen alone served as control. The procedure was repeated using leaves without pinpricks. Measurements of the lesion developed if any, were taken at 48 h interval till the leaves in control were covered by the infection. The per cent leaf area infection was calculated using the formula.

$$\text{Per cent leaf area infection} = \frac{\text{Length x breadth of lesion}}{\text{Length x breadth of the leaf}} \times 100$$

Since all the efficient isolates tested on detached leaves reduced the infection by more than 70 per cent when inoculated with pinpricks, all the 25 isolates were selected for evaluation of growth promoting ability.

3.5 EVALUATION OF ANTAGONISTIC ENDOPHYTES FOR GROWTH PROMOTION IN COCOA.

A pot culture experiment was laid out to assess the growth promoting effect of 25 efficient antagonistic endophytes selected from experiments 3.3 and 3.4. This experiment was conducted as two sets, *viz.*, using sterilized and non sterilized potting mixture in the ratio 1:1:1 at CCRP farm and in Green house at College of Horticulture, Vellanikkara, during June to October 2006.

The details of this experiment are as follows.

Design	: CRD
Number of treatments	: 26
Number of replication	: 5
Number of seedlings per replication	: 3
Cocoa clone used	: G VI 10 of CCRP Cocoa farm.
Method of application	: Seed treatment, soil drenching and foliar application at 15 and 30 days after sowing

The treatment details are given below.

Treatment	Isolate	Treatment	Isolate
T_1	EB-1	T_{14}	EB-52
T_2	EB-5	T_{15}	EB-53
T_3	EB-6	T_{16}	EB-60
T_4	EB-15	T_{17}	EB-61
T_5	EB-19	T_{18}	EB-62
T_6	EB-20	T_{19}	EB-64
T_7	EB-22	T_{20}	EB-65
T_8	EB-25	T_{21}	EB-67
T_9	EB-31	T_{22}	EF-72
T_{10}	EB-35	T_{23}	EF-78
T_{11}	EB-38	T_{24}	EF-80
T_{12}	EB-40	T_{25}	EF-81
T_{13}	EB-41	T_{26}	Control

3.5.1 Preparation of inoculum

Endophytic bacteria were multiplied on KBA plates. After 48h of incubation, the bacterial cells were taken and dispersed in sterile water so as to have 10^7cfu ml^{-1}. For fungal isolates (EF-72, EF-78, EF-81 and EF-82) the cultures were grown on PDA for seven days and the spore suspension (10^7cfu ml^{-1}) was made with sterile water.

3.5.2 Application of treatments and sowing

Cocoa beans freshly extracted from fully ripened pods were immersed in bacterial/spore suspension for 30 min. The beans were then sown in polythene bags of size 20cm x 15cm containing standard potting mixture. Cocoa beans treated with sterile water for 30min were sown in control. The beans were sown in poly bags of size 15 x 20 cm @ 20 beans distributed in 15 bags. After germination, excess seedlings were thinned out or transplanted to bags in which the beans failed to germinate. Second and third applications of endophytes were done at 15 and 30 days after sowing using the bacterial/spore suspension @ 50ml per bag as soil drench and foliar spray.

3.5.3 Observations recorded

Observations on germination percentage and biometric characters of seedlings *viz.*, seedling height and number of leaves, girth at collar region, root length and fresh and dry weight of shoot and root were recorded at periodical intervals.

3.5.3.1 Germination percentage

Observations on the beans germinated were taken beginning from one week after sowing to two weeks at two days interval.

3. 5.3.2 Biometric observations

Biometric observations were recorded for each seedling and average value was worked out. Height of the seedling and number of leaves per seedling were recorded at monthly intervals up to five months. Fresh and dry weight of shoot and root, girth at collar region and root length were recorded after five months of sowing.

3.5.4 Selection of potential endophytes

Data on germination percentage were compared separately for seedlings raised in sterilized and non sterilized potting mixture and data on biometric observations obtained from the four experiments were pooled and statistically analysed using DMRT. Based on the results of the above pot culture experiment, those isolates, which had maximum efficiency in augmenting various growth parameters, were selected for further evaluation. These include eight potential endophytic isolates of two bacteria, five fluorescent pseudomonads and one fungus.

3.6 MECHANISMS OF ACTION OF SELECTED ENDOPHYTES

The selected potential endophytic isolates *viz.*, EB-19, EB-22, EB-31, EB-35, EB-40, EB-41, EB-65 and EF-81 were further subjected to various analyses for studying the mechanisms by which they are producing antagonistic as well as growth promoting effect.

3.6.1 Ammonia Production

The qualitative estimation of production of ammonia was done following the method of Dye (1962) with slight modification. The selected endophytes were grown in 10 ml of peptone water (Appendix 1) and incubated at 30°C for four days. Three replications were maintained for each isolate. After incubation, 500 µl of Nesslers' reagent was added to the broth. Three replications were maintained for each isolate. The change in colour of the broth from faint yellow to deep yellow or brown colour indicated production of ammonia. The reaction was scored as nil, low, medium and high in 1-4 scale based on intensity of colour.

3.6.2 HCN Production

The method suggested by Wei *et al.* (1991) was used to detect whether the potential endophytes release hydrogen cyanide during their growth. Active growth of endophytes were inoculated to KBA plates supplemented with 4.4 g l^{-1} of glycine. Sterile filter paper strips soaked in picric acid solution (2.5 g picric acid + 12.5 g Na_2CO_3 in 1000 ml of water) were placed in the lid of each plate. Three replications were maintained for each isolate. The Petri plates were sealed with para film and incubated for 72 h. Change in colour of the filter paper strips from yellow to brown and to red indicates the production of HCN. The reaction was scored on a 1-4 scale depending on colour gradation.

3.6.3 Phosphate solubilization

The capacity of potential endophytes to solubilize tricalcium phosphate was tested *in vitro* using Pikovskaya's agar (Appendix 1) as well as in its broth (Pikovskaya, 1948). The isolates were spot inoculated at the centre of the plate containing the medium and incubated at 28°C for five days. Three replications were maintained for each isolate. Plates were observed for clearing zone around the colony and its diameter measured.

Pikovskaya's broth (10ml) inoculated with isolates was used for quantification of phosphate solubilization. The endophytic isolates were inoculated to 10 ml of

Pikovskaya's broth and incubated for 48 h. at 28°C at 150 rpm in an orbital shaking incubator. Liquid medium without inoculation served as control. The cultures were centrifuged at 7000 rpm for 10 min at 4°C, and then the supernatant was collected and precipitate discarded. One ml of supernatant was taken in a test tube and diluted by adding six ml of distilled water. Then two ml of chloromolybdic acid (to 15 g. of ammonium molybdate in 400ml warm distilled water, 342 ml of 12 N HCl was added, cooled and made up to one litre.) and one ml of chlorostannous acid (2.5g. of $SnCl_2$. H_2O in ml of conc. HCl, heated gently and volume made up to 100ml after cooling) were added to the mixture. Absorbance of the mixture was read at 660 nm using spectrophotometer (Thermospectronic-20).

Amount of soluble phosphate released from tricalcium phosphate by the endophytes were calculated from standard curve of P. The phosphate solubilizing capacity of the isolates was also scored following the scale based on the P solubilization as >1<5 mg $50ml^{-1}$ = 1; >5<10 mg $50ml^{-1}$ = 2; >10<14 mg 50 ml^{-1} = 3 and >14 mg $50ml^{-1}$ = 4.

3.6.4 IAA Production

Endophytic isolates inoculated in five ml of Kings'B broth supplemented with L-tryptophan @ 100 µg ml^{-1} (100 µg ml^{-1} L-tryptophan in 50 per cent ethanol), were incubated for 42 h. Growth of the isolates were removed by centrifugation at 5000 rpm for 10 min. One ml aliquot of supernatant was mixed thoroughly with four ml Salkowski's reagent and allowed to stand for 20 min. at room temperature. The absorbance was read at 535 nm. IAA concentration was calculated from standard curve. The isolates were also scored based on IAA production by the following the scale *viz.* IAA concentration >5<10 µg ml^{-1} = 1; >10<25 µg ml^{-1} = 2; >25<30 mg 50 ml^{-1} = 3 and >30 µg ml^{-1} = 4.

3.6.5 Determination of Antagonism Index

For comparing the antagonistic potential of the selected endophytes, their *in vitro* inhibitory effect against the pathogen was studied and a modified antagonism index (AI) suggested by Kasinathan (1998) was calculated, using the formula.

AI = PI x CB x TIME x IZ

AI = Antagonism Index

PI = Per cent Inhibition

CB = Colonization behavior

TIME = Time taken by the antagonist or pathogen to over grow after contact of antagonist or both

The isolates were scored based on AI using the scale; Score>1200<1500 = 1; >1500<1800 = 2; >1800< 2200= 3 and >2200 = 4

3.6.6 Determination of Vigour index

The selected potential antagonistic endophytes were assayed for growth promoting effect by method described by Shende *et al.* (1977), Elliot, and Lynch (1984).

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

The isolates were scored based on the scale, score>44<52 = 1; >52<60 = 2; >60< 68= 3 and >68 = 4

3.6.7 Determination of PGPE index

The selected antagonistic endophytes were evaluated for various attributes which make them potential bio control agents, as well as plant growth promoters. The qualitative and quantitative data of the parameters *viz.*, vigour index, antagonism index, HCN, ammonia, IAA production and phosphate solubilization were transformed into 1-4 scale and the PGPE index was calculated for each of the eight selected endophytes, which is originally suggested for comparing Plant Growth Promoting Rhizobacteria (Samanta and Dutta, 2004).

PGPE index = (Net PGPE score/Gross PGPE score) x 100

The isolates were scored using the scale; PGPI>25<45 = 1; >45<55= 2; >55< 60= 3 and >60 = 4

3.6.8 Selection of promising endophytes

Based on Vigour Index, PGPE Index and Antagonism index, the eight potential endophytes were again short listed to five promising endophytes *viz.*, EB-31, EB-35, EB-40, EB-65 and EF-81 which recorded higher values for the indices. These were subjected to further investigation on their capacity to produce siderophores, volatile and non- volatile inhibitory metabolites. Further, the mechanisms of induction of systemic resistance due to the application of promising endophytes were also studied.

3.6.9 Antibiosis test for production of volatile inhibitory metabolites

This test was carried out by slightly modifying the sealed Petri plate technique described by Dennis and Webster (1971). For this, two sterile Petri dish bases (90 mm) were taken, and for the fungal isolate (EF-81), molten cooled PDA was poured on both the dishes. One dish containing PDA, was inoculated with the fungal antagonist and allowed to grow for two days. For the bacterial antagonists, lawn of the candidate endophytic bacterium was prepared over the KBA on one Petridish by spread plate method. Ten mm disc of seven day old *Phytophthora* culture was placed at the centre of the other (PDA) dish. The two dishes were sealed together using parafilm and incubated at room temperature in such a way that the antagonist is in the lower dish. This allowed the volatile compounds produced by the antagonist to reach the pathogen growing in the upper dish. Similarly sealed dishes with pathogen inoculated on one and no antagonist on the other served as control. Three replications were maintained for each isolate. The radial growth of the pathogen was measured at 48 h interval up to seven days. Based on which per cent inhibition of the pathogen by endophytes was calculated, by comparing the same with growth of the pathogen in control.

3.6.10 Antibiosis test for production of diffusible, non volatile inhibitory metabolites

This test was carried out using cellophane paper method described by Dennis and Webster (1971). For this, cellophane paper (50µm thick) discs of 90 mm diameter were taken and sterilized in an autoclave at 121°C for 15min and then each sterilized disc was aseptically placed over PDA plates (90mm diameter). Ten mm discs taken from lawn/growth of each candidate endophytic isolate were placed at the centre of the cellophane paper and incubated for 72 h. After this, the cellophane paper along with the adhering antagonist was removed carefully and a ten mm disc of pathogen was immediately placed on the medium at central position previously occupied by the candidate antagonist. The radial growth of the pathogen was recorded at 48 h interval up to seven days and compared with its growth in control. Three replications were maintained and the per cent inhibition of the pathogen over control was calculated.

3.6.11 Detection of fluorescence

Log phase of the endophytic bacterial isolates were streaked on to KBA plate and incubated at 28°C for 48 h. The plates were observed on a UV trans illuminator to view the fluorescence (Kloepper, *et al.*, 1980a).

3.6.12 Iron dependant production of siderophores.

The four promising endophytic bacterial isolates were tested for iron dependant production of siderophores following standard protocol of Lopper (1988) and Kloepper, *et al.*, (1980b). King's B broth was amended with different concentrations of iron (0,100,200,300 µM $FeCl_3$). The medium was inoculated with 50µl. of log phase culture of bacterial isolates separately and incubated at 28°C for 72 h. The cells were pelleted by centrifugation at 7000rpm for 10 min and cell free culture filtrates (CFCF) were collected. The concentration of siderophore in the CFCF was read at 420nm.

3.7 INDUCTION OF SYSTEMIC RESISTANCE IN COCOA

A pot culture experiment was conducted during June, 2009 to October 2009 in the green house of the Department of Plant Pathology, College of Horticulture, to study the systemic resistance induced by the promising five endophytes against the pathogen in cocoa in comparison with standard cultures of *Pseudomonas fluorescens* (Pf_1 and Pf_2) and commonly used antifungal compounds (potasium phosphonate and Bordeaux mixture).

The details of the experiment are as follows.

Design	: CRD
Replication	: 3
Number of seedlings in each replication	: 10
Treatments	: 10

Treatment details

T_1	EB-31	T_6	*Pseudomonas fluorescens* (KAU)(Pf1)
T_2	EB-35	T_7	*Pseudomonas fluorescens* (TNAU) (Pf2)
T_3	EB-40	T_8	Potassium phosphonate 0.3 per cent
T_4	EB-65	T_9	Bordeaux Mixture 1.0 per cent
T_5	EF-81	T_{10}	Control

Method of application : The endophytes and standard cultures of *P. fluorescens* were applied as seed treatment and as soil drench and foliar application at 15 and 30 days after sowing. The chemicals were applied as soil application at the time of sowing and soil drench and foliar application at 15 and 30 days after sowing. Fifty treated cocoa beans were sown for each treatment, distributed in 30 bags. Later, 30 seedlings were maintained in each treatment for the study of induction of systemic resistance.

Data on germination percentage and biometric characters of seedlings were recorded as done in 3.5. Data on incidence and severity of seedling blight on challenge inoculation were also recorded at periodic intervals. Changes in total phenol, protein, defense related enzymes *viz.* peroxidase, polyphenoloxidase and β -1, 3 -glucanase were

quantitatively estimated using colourimetric methods at periodic intervals after challenge inoculation.

3.7.1 Challenge inoculation with the pathogen

The seedlings were challenge inoculated with the pathogen at two months after sowing. Inoculation was made on third leaf (light green coloured) from the top of the plant. Mycelial disc (10 mm) taken from seven day old culture of the pathogen grown on PDA was placed on the mid rib of the leaf after giving pinpricks. Small piece of cotton moistened with sterile water was placed over the mycelial disc. The inoculated seedling was then kept under polyethylene cover for 48h. Observations on percentage disease incidence and percentage disease severity were recorded at five and seven days after inoculation. The percentage disease severity was calculated using a 0–5 point scale (Tahi *et al.*, 2000), with zero indicating absence of symptoms and 1–5 increasing size of infected area (from 1 to 3) and increasing intensity of necrosis (from 4 to 5).

3.7.2 Assay of defence related compounds and enzymes

Defense related compounds such as phenol, protein, and enzymes such as peroxidase, polyphenol oxidase and β-1,3-glucanase were studied on the day of inoculation and at periodical intervals up to five days.

3.7.2.1 Total phenol content

Total phenol was estimated as per the procedure referred by Malick and Singh (1980). Leaf sample (500 mg.) was ground using pestle and mortar with 10 times volume of 80 per cent ethanol and centrifuged at 10,000 rpm for 20 min. The supernatant was saved and residue was re-extracted with five times volume of 80 per cent ethanol and centrifuged at 12000 rpm. Pooled the supernatants and evaporated to dryness. Dissolved the residue in 5.0 ml distilled water and pipetted out into test tubes at varying aliquots of 0.2-2.0 ml. Made up volume in each tube with distilled water and added 0.5 ml Folin Ciocalteu reagent. Incubated for 3.0 minutes and 2.0 ml of 20 per cent Na_2CO_3 solution was added. Mixed well and the tubes were placed in boiling

water for 1 minute, cooled and absorbance read at 650 nm. Concentration of phenol was calculated from standard curve of catechol.

3.7.2.2 Protein content

Total protein content was estimated as described by Lowry *et al.* (1951). One gram of leaf sample was extracted with 5.0 ml of 0.1 M sodium phosphate buffer, pH 7.5 using pre-cooled pestle and mortar and the extract was transferred to centrifuge tube. The homogenate was centrifuged (10000 rpm, 15 min 4°C). The supernatant was decanted, and discarded the residue. Equal volume of 15 per cent trichloro acetic acid (TCA) was added to the supernatant, which precipitated the protein. It was centrifuged at 3000 rpm. for 10 min. The supernatant was discarded and the precipitate dissolved in 0.1 N NaOH and the volume was made up to 10 ml with 0.1 N NaOH. This solution was used for protein assay.

From the above prepared sample, 0.5ml was pipetted out in to a test tube. Freshly prepared reagent comprising of 1.0 ml 0.1 per cent $CuSO_4.5H_2O$ + 1.0ml of 0.2 per cent sodium potassium tartarate + 100ml of 2 per cent Na_2CO_3 in 0.1N Na OH was added, mixed well and kept for 10 min. To this, 0.5 ml of 1N phenol reagent (2N Folin Cio calteau reagent diluted with equal volume of distilled water) was also added and the samples were vortexed thoroughly. The tubes were incubated at room temperature for 20-30min. The absorbance of samples was determined at 640nm (Thermospectronic-20) with a blank. A standard curve was prepared with different concentrations of bovine serum albumin (BSA) and used for calculation of protein content in the sample.

3.7.3 Assay of defence related enzymes

Two hundred mg fresh leaf sample from the leaf opposite to the challenge inoculated one was taken from each treatment and homogenized with 15 ml of suitable buffer for each enzyme in a pre-cooled pestle and mortar. The homogenate was centrifuged and the supernatant was stored at -20°C.

3.7.3.1 Assay of polyphenol oxidase activity

The activity of polyphenol oxidase was assayed using the method of Mayer *et al.* (1965). Enzyme extract was prepared in 0.1 M sodium phosphate buffer (pH 6.5) at 4°C. The reaction mixture contained 200µl enzyme extract and 2.6 ml of 0.1 M sodium phosphate buffer pH 6.5. The reaction was initiated by adding 200 µl 0.01 M catechol. The change in absorbance at 420 nm was read immediately and at 30 S intervals till 5 min. The enzyme activity was expressed as change in absorbance by PPO activity min^{-1} mg^{-1} of fresh tissue.

3.7.3.2 Assay of peroxidase activity

Peroxidase activity was assayed using the procedure referred by Sadasivam and Manikkam (1996). Fresh leaf sample (200 mg) was homogenized with 15 ml 0.3 M phosphate buffer pH 7. The homogenate was centrifuged at 20,000 g for 20 min at 4°C and the supernatant was used for further analysis. The reaction mixture contained 500 μl enzyme extract, 1.0 ml of 45 nM guiacol, 1.0 ml 0.3 M sodium phosphate buffer and 1.0 ml H_2O_2 22.5 mM. Absorbance at 436 nm was read immediately after adding the enzyme extract. Change in absorbance was read at 30 S intervals for 3 min.

3.7.3.3 Assay of β -1, 3 - glucanase activity

Basic procedure of Pan *et al.* (1991) with slight modification by Parab (2000) was used for assay of the β 1, 3 - glucanase activity in cocoa. Leaf extract was prepared from 500 mg fresh leaf sample (made into small pieces and frozen at -80°C). Homogenized in chilled mortar and pestle with 10 ml of sodium acetate buffer 0.05 M, pH 5. Added 500μl of ascorbic acid (5nm) PMSF (0.1 M), β-mercapto ethanol and 1000μl of Cystein HCl (0.05 M) to the above paste and mixed well. The resulting mixture was taken into 30 ml centrifuge tubes and centrifuged at 15000 rpm for 10 min at 4°C. The supernatants were collected in separate eppendorf tubes and these served as crude enzyme extracts.

Enzyme extract (62.5 μl) was mixed with equal volume of laminarin solution (4 per cent). Incubated at 40°C for 10 min. 375 μl of di nitro salycilic acid was added to

stop the reaction and heated for 5 min. in boiling water bath. Chilled for 5 min in ice bath when the colour changed from yellow to brown red, it was diluted with distilled water 4.5 ml, vortexed and read the absorbance at 500 nm.

3.7.4 Isozyme analysis

Fresh cocoa leaf sample 200mg taken after challenge inoculation was homogenized with 0.1M. sodium phosphate buffer (pH. 7.0) using a pre-cooled mortar and pestle (Malick and Singh, 1980). The homogenate was centrifuged at 15000rpm at 4°C for 15 min. The isozymes were analyzed as per the protocol of Laemmli (1970) by Native Poly Acrylamide Gel Electrophoresis (Native PAGE).

The electrophoresis was carried out with a Bio-Rad vertical electrophoresis unit. (Bio-Rad, USA). The resolving gel mixture (8 per cent) (Appendix II) was prepared and poured between the glass plates. A layer of distilled water was added above the gel layer and was allowed to polymerize for 30 min. Stacking gel mixture (6 per cent) (Appendix II) was prepared and poured over the separating gel after removing the layer of water. The comb was placed in the stacking gel and allowed to set for 30 min. After polymerizing, the gel was installed in the electrophoresis apparatus. The electrode buffer (Appendix II) was poured slowly. The centrifuged supernatant (45 μl.) of different treatment samples were taken and mixed with the sample buffer (5 μl) (Appendix II) and finally 50 µl was loaded carefully into the wells with a micropipette. The electrophoresis was carried out at the constant current of 15mA for 15 min. or more until the samples moved through the stacking gel and then at 30mA for about 2-3h. After the completion of the running, the gel was carefully removed and immersed in the staining solutions with uniform shaking. It was then destained by using destaining solutions known for each enzyme to visualize the bands. The electrophorogram was photographed.

3.7.4.1 Peroxidase

The bands of isoforms of peroxidase on the gel after electrophoresis were visualized by incubating the gel 37°C for 20-30 min. in the following staining solution.

Solution A: 1. O dianicidine 0.05g. in 1.0ml HCl (1 N)

2. Sodium acetate buffer (pH 5.4) 3ml

3. Distilled water 26ml.

Solution B: 0.01 per cent H_2O_2.

The gel was incubated in solution A for 30 min. at 37^{0}C. The solution B was poured into it. Orange red coloured bands of peroxidase isoforms were observed. The reaction was arrested by adding seven per cent acetic acid and the electrophorogram was photographed.

Analysis of isozyme bands were designed and dendrogram was constructed based on the isozyme profiles of PO and PPO induced by various treatments using the unweighed pair group method of arithmetic average (UPGMA) with NTSYS package.

3.7.4.2 Polyphenol oxidase

The electrophorogram of polyphenol oxidase was visualized after incubation of the gel for 30 min. in 0.1 per cent p-phenylene diamine in 0.1M sodium phosphate buffer (pH 7.0) followed by 10mM catechol in the same buffer (Jayaraman, *et. al.*, 1987).The enzyme reaction was stopped as described in 3.8.1 and the Native PAGE profile was photographed.

3.8 FIELD EVALUATION OF SELECTED PROMISING ENDOPHYTES AGAINST *Phytophthora* POD ROT

An experiment was conducted during June to September 2008 in the existing cocoa garden at the CCRP cocoa farm in order to evaluate the selected promising endophytes in suppression of the disease under field condition. The details of the field experiment are given below.

Design	-	RBD
Replication	-	Three 15 year old plants

Treatments - 10

Treatment details

T_1	EB-31	T_6	*Pseudomonas fluorescens*, KAU (Pf_1)
T_2	EB-35	T_7	*Pseudomonas fluorescens*, TNAU (Pf_2)
T_3	EB-40	T_8	Bordeaux mixture 1.0 per cent
T_4	EB-65	T_9	Potassium phosphonate 0.3 per cent
T_5	EF-81	T_{10}	Control

Method of application - Spray on the pods, trunk and pod bearing branches thrice at monthly interval.

3.8.1 Preparation of inoculum

The four selected promising endophytic bacterial isolates and standard cultures of *P. fluorescens* were mass multiplied on Kings' B agar plates. After incubating for 48h, the bacterial lawn was scraped out and cell suspension was prepared by adding sterile water so as to get 10^8 cfu ml^{-1} and used for spraying. The fungal isolate was grown on PDA plates for seven days. Spore suspension was made by adding sterile water, filtered through double layered muslin cloth in order to remove mycelial bits. Concentration of the spore suspension was adjusted to 10^8 cfu ml^{-1}. The treatments were applied thrice @500ml per plant at monthly intervals beginning from the second week of June at the onset of monsoon.

3.8.2 Observations recorded.

Observations on the total number of pods on each tree and the number of infected pods were recorded at weekly intervals beginning from the day of first spraying till three weeks after the third spraying and the percentage incidence of pod rot was calculated.

3.9 CHARACTERIZATION AND IDENTIFICATION OF PROMISING ENDOPHYTES.

3.9.1 Characterization of promising bacterial endophytes

Characterization of different promising bacterial antagonists *viz.*, EB-31, EB-35, EB-40 and EB-65, was carried out following the methods as suggested in the Manual of

Microbiological Methods, published by the Society of American Bacteriologists (1957) and also by the Bergy's manual of systematic Bacteriology, Vol. 1 (Stanley *et. al.* 1989). The cultural, morphological, physiological and biochemical characters of the isolates such as, colony characters, Gram's reaction, pigment production, oxidase and catalase reaction, arginine hydrolase, lipase and levan production, gelatin liquefaction, starch hydrolysis, denitrification, citrate utilization, lysine decarboxilase, ornithine decarboxilase, urease test, phenylalanine deamination, nitrate reduction, H_2S production and mode of utilization of sugars *viz.*, glucose, fructose, lactose, sucrose, arabinose, cellobiose, mannose, sorbitol, dulcitol, meso-inositol and adonitol were studied along with the reference cultures of *P. fluorescens* (Pf_1 and Pf_2). The Hi Assorted TM Biochemical test kit for Gram negative rods were also employed for characterization of the endophytic isolates and compared with the Interpretation chart given in the manual. Three replications were maintained for each isolate.

3.9.2 Identification of the endophytic fungal isolate

The cultural and morphological characters of the fungal isolate was studied for identification. Further, confirmation of the identification of the fungus was done at the Indian Type Culture Collections (ITCC), Division of Mycology and Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi.

3.10 MOLECULAR CHARACTERIZATION OF PROMISING ENDOPHYTIC BACTERIAL ISOLATES

The promising endophytic bacterial isolates *viz.*, EB-31, EB-35, EB-40 and EB-65 were subjected to molecular characterization in order to identify them up to the species level. The aim of this study was to confirm the results of biochemical characterization already done in (3.9.1). The bacterial isolates were characterized by 16SrDNA sequencing. This experiment was done at the Molecular Plant Pathology laboratory at the Rubber Research Institute of India, Kottayam.

3.10.1 Isolation and purification of genomic DNA

The procedure suggested by Pitcher *et al.* (1989) was followed for isolation of genomic DNA from the promising bacterial endophytes. The bacteria were grown in tryptic soy broth (Hi media code No. M011), for 24 hrs in pure culture. The cells were removed by centrifuging at 5000 rpm for 10 min. The supernatant was removed and cells pelletted in 1.0 ml of re suspension buffer. The cells are pelletted again (5000 rpm, 10 min) removed the supernatant and 100 μl TE buffer (100x) (Appendix III) was added and mixed using a pipette. Added 500 μl of GES solution (Guanidine thiocyanate–EDTA-Sarkosyl) (Appendix III) and mixed gently. Incubated on ice for 5 minutes. Added 250 μl of cold (-20°C) Ammonium acetate (7.5 m) and mixed by gently shaking the tubes. The tubes were again incubated on ice for 5 min. Added 500 μl of chloroform iso amyl alcohol (24:1) and shaken vigorously until the solution became homogeneously milky. Centrifuged at 10,000 rpm, 10 min or until the upper phase is clear. Carefully removed 700 μl of DNA solution from the upper phase using 1000 μl tip and added to pre cooled (-20°C) tubes containing 378 μl of isopropanol. The tubes were shaken gently until white cloud of DNA precipitate became visible. Centrifuged 98000 rpm, 10 min) and removed supernatant. Added 150 μl 70% ethanol slowly without mixing. Centrifuged briefly and removed ethanol with 200 μl pipette. Repeated centrifugation and removed residual ethanol. The DNA pellet was air dried and redissolved in 200 μl TE pH 8, incubated at room temperature until DNA is dissolved. 25 μl RNA are (250 μg ml^{-1}) was added and mixed gently. Incubated for one hour at 37°C and stored at 4°C. The concentration of DNA was determined by spectrophotometry and adjusted the concentration to 50 ng. ml^{-1}. The quality of DNA was assessed by agarose gel electrophoresis (Sambrook *et al.* (1989) and DNA was visualized under UV. The DNA bands were documented under gel documentation system and stored. Bacterial Genomic DNA was isolated from endophytic bacteria EB-31, EB-35, EB-40 and EB-65.

3.10.2 PCR amplification of 16S rDNA

The genomic DNA isolated from the most promising endophytic bacteria from cocoa were subjected to PCR amplification of 16SrDNA with the intention to identify them. The following conserved eubacterial 16SrDNA primers were used for the amplification.

1. 8F – [AGAGTTTGATCCTGGCTCAG]
2. 1492r – [TACGGTACCTTGTTAGCACTT]

PCR amplification was performed with 50 ng μl^{-1} of genomic DNA. Composition of the reaction mixture was as follows:

Stock solution	**μl per one 20 μl reaction**
Buffer 5x	5.0
dNTP mix (2 mM)	2.5
Taq DNA polymerase (3 U μl^{-1})	0.3
DNA 50 ng	1.0
Primer (8F + 1492r)	1.0 each
Sterile distilled water	12
Total	20 μl

The PCR reaction was carried out in a Bio-Rad Thermal Cycler with the following cycle

Reaction	Temperature (°C)	Time (min)	Number of cycles
Initial denaturation	95	1	1
Denaturation	94	1	35
Annealing	55	1	35
Extension	72	2	35
Final extension	72	3	1

The amplicons were subjected to agarose gel electrophoresis on 1.5% gel in 0.5 x TAE Buffer (Appendix III). Direct load wide range DNA marker (Sigma D7058) having 1600 bp (equivalent to 16SrDNA) was loaded along with the amplicons. The DNA bands were visualized in a gel documentation system under UV and stored.

3.10.3 Elution of amplified products from agarose gels (Min Elute™ Gel extraction kit – Qiagen, Cat.No.28604)

The gel with the amplicons was viewed over long wave length UV radiation quickly and the DNA band was excised from the agarose gel with a clean sharp scalpel. The gel slice was weighed in a colourless eppendorf tube. Three times its volume of the QG buffer (provided with the kit) was added to the gel and incubated at 50^0C for 10 min or until the gel slice was completely dissolved in the buffer. The tube was vortexed every 2-3 min. in order to dissolve the gel during incubation. After the gel slice has dissolved completely, the colour of the mixture was yellow. Then one gel volume of isopropanol was added to the tube and mixed with the sample. A MinElute spin column was placed in a two ml collection tube. The sample was applied to the MinElute column and was centrifuged at 12000rpm for one minute. The flow through was discarded and the column was placed back in the same tube to which, 0.5 ml of buffer QG was added, centrifuged at 12000rpm for one minute. The flow through was discarded and the MinElute column was placed in the same tube. Buffer PE(750µl) (provided with the kit) was added with which the MinElute column was washed and was centrifuged for one minute. Again the flow-through was discarded; the column was centrifuged for an additional one minute at 13000rpm. Then the column was placed in a clean 1.5 ml micro centrifuge tube. To elute DNA, 10 μl water was added to the center of the membrane. The column was allowed to stand for one minute and then centrifuged for one minute. The purified DNA was stored at -20^0C.

3.10.4 Ligation

The InsTAclone TM PCR cloning kit (Fermentas, Cat.No. #K1214) with the TA cloning vector pT57R /T was used for cloning purified PCR products. A vector to insert ratio of 1:3 was used for ligation.

The ligation mixture was prepared as follows.

Component	Volume
Vector pTZ57R/T(o.18pmol ends)	3 μl
5X ligation buffer	6 μl
PCR product (0.54pmol ends)	2 μl

Nuclease free water	To 29 μl
T4 DNA Ligase	1 μl
Total volume	30 μl

The ligation mixture was vortexed briefly and centrifuged for three to five seconds. The mixture was then incubated at 22^0C for one hour.

3.10.5 Cloning of ligated product in *E coli* as per Fermentas kit (InsTAcloneTM PCR cloning kit #K1213)

Transformation was done as follows. A loopful of logphase bacterial culture was added to 1.5ml pre-warmed C-medium. The cells were suspended by gentle mixing and incubated for two hours at 37^0C with continuous shaking. The cells were pelleted by centrifugation for one minute and the supernatant was discarded. The cells were re-suspended in 300 μl of T-solution and incubated on ice for five minutes. Again it was centrifuged for one minute and supernatant was discarded. The pelleted cells were resuspended in 120 μl of T-solution and incubated on ice for five minutes.

The ligation mixture was dispersed @ five μl each into micro-centrifuge tubes and chilled on ice for two minutes. Competent cells prepared earlier was added to each tube containing DNA, mixed and incubated on ice for five minutes. The cells were spread over pre-warmed Luria Broth (LB) plate containing 50 μg ml^{-1} ampicillin coated with 40 μl X-gal (20mg ml^{-1}) and four μl IPTG (200mg ml^{-1}). The plates were incubated overnight at 37^0C. Transformants containing the insert were selected by blue/white screening.

3.10.6 Sequencing of PCR amplified 16SrDNA.

The transformed cells containing amplified fragments were sent for sequencing at Macrogen, Korea. The sequence data for 16SrDNA of the promising isolates were blasted in Ribosomal data project release 10 and the sequence homology was compared.

3.11 RADIOTRACER STUDY ON ENTRY AND ESTABLISHMENT OF GROWTH OF ENDOPHYTES WITHIN THE PLANT

This experiment was conducted to understand whether the four promising endophytes gain entry into the cocoa plants and established within. The experiment was

conducted at Radiotracer Laboratory, College of Horticulture, Vellanikkara. The radioisotope ^{32}P was procured from the Board of Radiation and Isotope Technology, Mumbai. Since the radioisotope (^{32}P) was to be used within one week after receipt, the promising bacterial endophytes were chosen for this study as bacteria can be easily multiplied and labeled.

3.11.1 Preparation of radio labeled inoculum

Pure cultures of the promising bacterial endophytes were prepared on solid media *viz.*, King's B agar for the three fluorescent pseudomonads *viz.*, EB-31, EB-40 and EB-65 and Nutrient agar for EB-35 (*Bacillus subtilis*). Log phase cultures from these plates were used for radiolabeling. For preparing labeled inoculum, 41 μCi ^{32}P ml^{-1} was added to sterilized liquid medium (King's B). One loop full of the log phase culture was aseptically transferred to 50 ml of the labeled liquid medium and incubated for 48 h. at 28°C.

3.11.2 Centrifugation

Radio activity present in the growth medium was removed by centrifugation before plant inoculation in order to assure that the radio activity that may be detected in inoculated plant is from the bacteria which had entered into it. The broth culture was centrifuged at 5000 rpm for 15 min. The supernatant was removed and the bacteria pelletted at the bottom of the centrifuge tube was resuspended in sterile water and centrifuged again. This was repeated thrice and the supernatant was tested for presence of radioactivity in a scintillation unit. Centrifugation and resuspension in fresh sterile water was repeated until the supernatant was free of radioactivity. Finally the labeled bacterium was resuspended in 25 ml sterile water and used for plant inoculation.

3.11.3 Plant Inoculation

Inoculation of cocoa seedlings with labeled bacteria was done in three different sites, *viz.* root inoculation, foliar application and inoculation on the stem. In all the three methods, inoculation was done without giving injury.

3.11.3.1 Root inoculation

Bacterial suspension (1.5 ml) was taken in small plastic tubes. Actively growing feeder root was carefully excavated, without breaking. The tip of the root washed to remove adhering soil and was placed carefully in the bacterial suspension contained in a small plastic tube.

3.11.3.2 Foliar application

Bacterial suspension (500 μl) was applied on the upper surface of the third leaf of a new flush as small droplets using micro pipette, and allowed to dry.

3.11.3.3 Inoculation on the stem

On the stem, cotton wet with 500 µl bacterial suspension was placed at the collar region of the stem.

3.11.3.4 Inoculation on pods

Immature cocoa pods collected from CCRP farm were used. Bacterial suspension (500 µl) was carefully applied into the depression around the pedicell using micropipette and allowed to air dry. Three replications were maintained for each bacterium.The inoculated plants and pods were kept in the green house attached to RTL, for two days, in order to allow the labelled bacteria to penetrate and colonize the tissues.

3.11.4 Preparation of the plant parts for autoradiography

Two days after inoculation, the aerial plant parts were detached by cutting at the base of the stem in the case of root and leaf inoculated plants. In the case of plants inoculated on the stem, it was cut at three cm above the point of inoculation. The inoculated part was cut and removed from the pods. Then the pods were cut horizontally into two halves using a sharp knife. Three thin slices were then taken one from the centre, and from two sides.

The plant parts were then arranged on absorbent paper in their original position, labelled and secured with adhesive tape. The specimens sandwiched between absorbent sheets were then pressed in herbarium press and allowed to dry at room temperature. Specimens cut from pods were dried in electric oven for two days at 50°C and then air dried.

3.11.5 Autoradiography

After drying, the pressed specimens were autoradiographed by placing on X-ray films in dark and covered with smooth paper and pressed. The X-ray films were exposed for 10 days in the press. The plant parts were removed and the film was developed using a commercial X-ray film developer solution.

3.12 STATISTICAL ANALYSIS.

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

Results

4. RESULTS

The studies on "Endophytic microorganism mediated systemic resistance in cocoa against *Phytophthora palmivora* (Butler) Butler" were carried out at the Department of Plant Pathology, College of Horticulture, Vellanikkara and cocoa farm of Cadbury-KAU Co-Operative Cocoa Research Project (CCRP), attached to College of Horticulture during 2005-2010. The results of the study are presented below.

4.1 ISOLATION AND CHARACTERIZATION OF THE PATHOGEN

4.1.1 Isolation of the pathogen

The pathogen causing *Phytophthora* pod rot of cocoa was isolated from naturally infected cocoa pods collected from CCRP cocoa farm, College of Horticulture, Vellanikkara (Plate 4.1). The isolate was purified by hyphal tip method and maintained on PDA slants by periodic sub culturing. Pathogenicity of the organism was proved by inoculation on healthy cocoa pods as mentioned in 3.1. The isolate produced circular water soaked lesions within 48h. Later the lesions enlarged and turned into chocolate brown colour. Whitish growth of the pathogen consisting of mycelia and sporangia was produced over the dark brown area immediately behind the advanced border. Reisolation from artificially inoculated pods yielded organism having the same characters as the original one.

4.1.2 Cultural and morphological characters of the pathogen

The mycelium of the isolate was branched, hyaline and coenocytic. The somatic hyphae were 3.5 - 5 μm in breadth. Sporangiophores developed from somatic hyphae and their tip became swollen, later developed into sporangia. Sporangiophores were indeterminate and measured 46 - 129 μm in length. Sporangia were spherical when young, with less dense protoplasm. Mature sporangia were typically pear shaped with small, but prominent papilla (Plate 4.2). The sporangia were borne terminally on the sporangiophore in a simple, sympodial fashion and were caducous. Sporangia measured

Plate 4.1 *Phytophthora* pod rot of cocoa

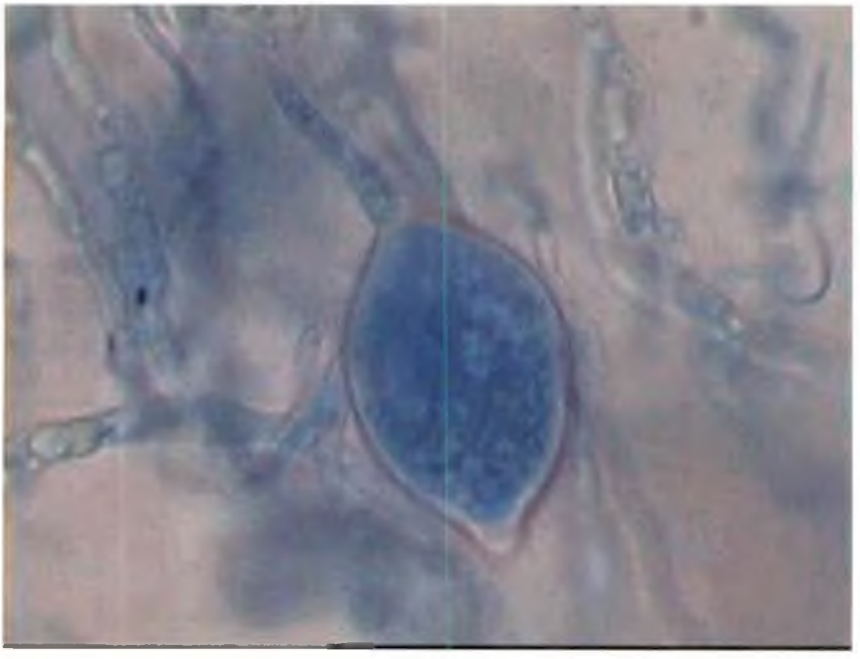

Plate. 4.2. Mature sporangium of *Phytophthora palmivora*

26.2-58.5 x 18-35 μm at maturity. Deciduous sporangia had short and thick pedicel, its length ranged from 3-4 μm. The average L/B ratio of sporangia was 1:15.

4.2 ISOLATION AND ENUMERATION OF ENDOPHYTIC MICROFLORA FROM COCOA

Fifteen samples each of roots, leaves, shoots and pods of cocoa were collected during the months of June-September 2005 from major cocoa growing areas of Kottayam, Idukki, Pathanamthitta, Thrissur and Palakkad districts. The total endophytic microflora *viz.*, fungi, bacteria, actinomycetes, yeasts and fluorescent pseudomonads were quantitatively estimated by trituration followed by serial dilution plating. Actinomycetes could not be isolated from any one of the samples collected.

4.2.1 Endophytic fungi

Data presented in Table 4.1 reveals that population of endophytic fungi varied among different locations as well as plant parts ranging from zero to 14.5 x 10^4cfu g^{-1} of plant tissue. More fungi were present in roots than in other parts of the plant (3.7 x 10^4cfu g^{-1} of plant tissue). The least count of fungi were found in samples of shoot collected from different locations (0.4 x 10^4 cfu g^{-1} plant tissue). Endophytic fungal population was higher in samples collected from Palakkad and Thrissur districts than in other locations. The maximum number of fungi were present in roots collected from Palakkad, whereas no fungi could be obtained from that from Pathanamthitta. Shoots and pods collected from Pathanamthitta also harboured no fungi. Similarly, shoots and leaves from Kottayam and Idukki as well as pods from Palakkad also yielded no fungi. Cocoa pods from Thrissur recorded the highest number of fungi (2.8 x 10^4 cfu g^{-1} plant tissue). Among leaf and shoot samples also the highest count of fungi was obtained from Thrissur.

4.2.2 Endophytic bacteria

Endophytic bacteria in varying numbers (1.9 to 50.8 x 10^7cfu g^{-1} plant tissue) were obtained from the samples collected (Table 4.2). In general, roots contained more

bacteria than other parts of the plant. The count of bacteria was the highest in roots (50.8 x 10^7 cfu g^{-1} plant tissue) collected from Pathanamthitta, followed by that from Palakkad (41.5 x 10^7 cfu g^{-1} plant tissue). The least number of bacteria was present in pods (8.2 x 10^7cfu g^{-1} plant tissue). Among different locations, bacterial population was the highest in Thrissur (20.6 x 10^7cfu g^{-1} plant tissue) and the lowest in Palakkad (13.1 x 10^7 cfu g^{-1} plant tissue). However, among the different samples studied, the least number of endophytic bacteria (1.9 x 10^7cfu g^{-1} plant tissue) was obtained from cocoa pods collected from Pathanamthitta district.

4.2.3 Endophytic yeasts

Endophytic yeasts could be isolated from all the samples studied (Table 4.3). Among different samples, population of yeasts varied from 0.7 x 10^4cfu g^{-1} to 26 x 10^4cfu g^{-1}. Unlike fungi and bacteria, the mean population of yeasts in general was more in cocoa pods than other parts. However, it was the least in leaves (7.9 x 10^4 cfu g^{-1} plant tissue) and the leaves from Idukki recorded more yeasts than other locations. Among different locations, more number of yeasts were recorded in samples from Kottayam district whereas the minimum was from that of Thrissur. However, the highest count was obtained from shoots collected from Kottayam district (26 x 10^4cfu g^{-1}), followed by pods from Pathanamthitta (24.7 x 10^4 cfu g^{-1}) whereas pods from Palakkad yielded the lowest population of yeasts.

4.2.4 Fluorescent pseudomonads

Results depicted in Tables 4.1 to 4.4 shows that among the endophytic microbes present in cocoa, fluorescent pseudomonads were predominant. Their population ranged from 2.0x10^7 cfu g^{-1} to 58.3x10^7cfu g^{-1} plant tissue. Similar to bacteria and fungi, they were also more in roots (35.3x10^7cfu g^{-1}) than in other parts studied. Among different locations, the count of fluorescent pseudomonads was more in Thrissur (23.3x10^7cfu g^{-1}) followed by Palakkad (20.2x10^7cfu g^{-1}). The least number of fluorescent pseudomonads was present in pods (8.5x10^7 cfu g^{-1}). Roots collected from Pathanamthitta yielded the

Table 4.1 Population of endophytic fungi in cocoa plants at different locations

Sl. No.	District	Fungi ($x10^4$cfu g^{-1} plant tissue)*				
		Root	Shoot	Leaf	Pod	Mean
1.	Kottayam	0.7	0	0	2.3	0.8
2.	Idukki	0.3	0	0	1.3	0.4
3.	Pathanamthitta	0	0	0.7	0	0.2
4.	Thrissur	3.4	1.5	1.4	2.8	2.3
5.	Palakkad	14.5	0.7	0.7	0	4.0
Mean		3.7	0.4	0.6	1.3	

*Average of three locations

Table 4.2 Population of endophytic bacteria in cocoa plants at different locations

Sl. No.	District	Bacteria ($x10^7$cfu g^{-1} plant tissue)*				
		Root	Shoot	Leaf	Pod	Mean
1.	Kottayam	13.9*	17.3	19.9	12.2	15.8
2.	Idukki	16.3	6.7	19.8	12.9	13.9
3.	Pathanamthitta	50.8	2.9	3.4	1.9	14.8
4.	Thrissur	28.9	20.3	22.8	10.5	20.6
5.	Palakkad	41.5	4.3	3.0	3.7	13.1
Mean		30.1	10.3	13.8	8.2	

*Average of three locations

Table 4.3 Population of endophytic yeasts in cocoa plants at different locations

Sl. No.	District	Yeasts (x 10^4cfu g^{-1} plant tissue)*				
		Root	Shoot	Leaf	Pod	Mean
1.	Kottayam	10.0	26.0	11.3	13.7	15.3
2.	Idukki	17.7	11.3	16.0	14.3	14.8
3.	Pathanamthitta	2.1	3.3	5.4	24.7	8.9
4.	Thrissur	1.7	2.3	2.9	2.5	2.4
5.	Palakkad	8.7	3.6	3.7	0.7	4.2
Mean		8.0	9.3	7.9	11.2	

*Average of three locations

Table 4.4 Population of endophytic fluorescent pseudomonads in cocoa plants at different locations

Sl. No.	District	Fluorescent pseudomonads (x10^7cfu g^{-1} plant tissue)*				
		Root	Shoot	Leaf	Pod	Mean
1.	Kottayam	8.7	21.3	14.0	10.7	13.7
2.	Idukki	19.0	8.3	14.3	11.7	13.3
3.	Pathanamthitta	58.3	2.0	4.0	3.7	17.0
4.	Thrissur	43.3	14.3	21.4	14.2	23.3
5.	Palakkad	47.0	28.3	3.3	2.0	20.2
Mean		35.3	14.8	11.4	8.5	

*Average of three locations

maximum number of fluorescent pseudomonads (58.3 x 10^7 cfu g^{-1}), followed by roots from Palakkad (47.0 x 10^7cfu g^{-1}).

4.3 *IN VITRO* ANTAGONISTIC EFFECT OF ENDOPHYTES AGAINST THE PATHOGEN

4.3.1 Preliminary screening of endophytes

Cocoa samples collected from various locations yielded 325 isolates of endophytes, which include 116 bacteria, 153 fluorescent pseudomonads, 34 yeasts and 22 fungi. They were subjected to preliminary screening as per 3.3.1 in order to test the antagonistic property towards the pathogen (Plate 4.3). Out of the 325 isolates, 243 did not exhibit any antagonism towards the pathogen whereas, 82 were found to be antagonistic in varying degrees. These isolates included 28 bacteria, 29 fluorescent pseudomonads, 21 yeasts and four fungi. The details of these antagonistic endophytes are presented in Table 4.5.

4.3.2 *In vitro* evaluation of antagonistic endophytes

The 82 endophytes, which were found exerting antagonism towards the pathogen in the preliminary screening, were subjected to further evaluation by dual culture method (Plate 4.4). This was done to evaluate the extent of antagonism and to select the ones that are more efficient. Data on the per cent inhibition of the pathogen by the antagonists (Table 4.6) revealed that, the extent of inhibition varied among isolates, with the maximum of 84 per cent by the isolate EB-53 followed by an inhibition percentage of 83.1, 82.2 and 82.1 by isolates EB-14, EB-31, EB-2 and EB-1 respectively. Of the remaining, 18 isolates recorded inhibition between 70-80 per cent, seven between 60-70 per cent and eight between 50-60 per cent inhibition. Forty to fifty per cent inhibition was exhibited by six isolates and 38 gave less than 40 per cent inhibition. However, 20 isolates recorded very low *i.e.*, less than 10 per cent inhibition only.

Table 4.5 Details of the antagonistic endophytes isolated from cocoa

Sl. No.	Isolate	District	Part of the plant	Medium of isolation	Type of organism
1.	EB-1	Thrissur	Root	KBA*	Fluorescent Pseudomonad
2.	EB-2	Thrissur	Root	NA**	Bacterium
3.	EB-3	Idukki	Pod	NA	Bacterium
4.	EB-4	Idukki	Leaf	KBA	Fluorescent Pseudomonad
5.	EB-5	Kottayam	Pod	NA	Bacterium
6.	EB-6	Thrissur	Pod	KBA	Fluorescent Pseudomonad
7.	EB-7	Thrissur	Leaf	KBA	Fluorescent Pseudomonad
8.	EB-8	Kottayam	Pod	GYEPA***	Yeast
9.	EB-9	Thrissur	Leaf	GYEPA	Yeast
10.	EB-10	Thrissur	Root	NA	Bacterium
11.	EB-11	Thrissur	Root	GYEPA	Yeast
12.	EB-12	Kottayam	Pod	KBA	Bacterium
13.	EB-13	Thrissur	Pod	NA	Bacterium
14.	EB-14	Kottayam	Pod	NA	Bacterium
15.	EB-15	Idukki	Root	KBA	Bacterium
16.	EB-16	Idukki	Leaf	KBA	Fluorescent Pseudomonad
17.	EB-17	Thrissur	Pod	NA	Bacterium
18.	EB-18	Thrissur	Root	GYEPA	Yeast
19.	EB-19	Thrissur	Leaf	KBA	Fluorescent Pseudomonad
20.	EB-20	Thrissur	Shoot	KBA	Fluorescent Pseudomonad
21.	EB-21	Thrissur	Shoot	GYEPA	Yeast
22.	EB-22	Thrissur	Pod	NA	Bacterium
23.	EB-23	Thrissur	Root	GYEPA	Yeast
24.	EB-24	Thrissur	Leaf	GYEPA	Yeast
25.	EB-25	Thrissur	Leaf	KBA	Fluorescent Pseudomonad
26.	EB-26	Kannara	Leaf	GYEPA	Yeast
27.	EB-27	Kannara	Root	NA	Fluorescent Pseudomonad
28.	EB-28	Thrissur	Pod	GYEPA	Yeast
29.	EB-29	Idukki	Shoot	NA	Bacterium
30.	EB-30	Idukki	Shoot	KBA	Fluorescent Pseudomonad
31.	EB-31	Thrissur	Pod	KBA	Fluorescent Pseudomonad
32.	EB-32	Palakkad	Shoot	NA	Bacterium
33.	EB-33	Palakkad	Shoot	KBA	Fluorescent Pseudomonad
34.	EB-34	Kottayam	Shoot	KBA	Fluorescent Pseudomonad
35.	EB-35	Idukki	Pod	NA	Bacterium
36.	EB-36	Kottayam	Pod	KBA	Fluorescent Pseudomonad
37.	EB-37	Idukki	Root	KBA	Fluorescent Pseudomonad
38.	EB-38	Kottayam	Pod	NA	Bacterium
39.	EB-39	Kottayam	Leaf	GYEPA	Yeast
40.	EB-40	Thrissur	Pod	KBA	Fluorescent Pseudomonad
41.	EB-41	Idukki	Pod	KBA	Fluorescent Pseudomonad

Sl. No.	Isolate	District	Part of the plant	Medium of isolation	Type of organism
42.	EB-42	Thrissur	Leaf	GYEPA	Yeast
43.	EB-43	Thrissur	Root	GYEPA	Yeast
44.	EB-44	Palakkad	Shoot	GYEPA	Yeast
45.	EB-45	Palakkad	Shoot	GYEPA	Yeast
46.	EB-46	Palakkad	Root	NA	Bacterium
47.	EB-47	Palakkad	Leaf	KBA	Bacterium
48.	EB-48	Palakkad	Leaf	KBA	Bacterium
49.	EB-49	Palakkad	Shoot	GYEPA	Yeast
50.	EB-50	Palakkad	Shoot	GYEPA	Yeast
51.	EB-51	Palakkad	Shoot	GYEPA	Yeast
52.	EB-52	Palakkad	Shoot	KBA	Fluorescent Pseudomonad
53.	EB-53	Palakkad	Root	KBA	Fluorescent Pseudomonad
54.	EB-54	Palakkad	Shoot	KBA	Fluorescent Pseudomonad
55.	EB-55	Palakkad	Root	GYEPA	Yeast
56.	EB-56	Palakkad	Root	GYEPA	Yeast
57.	EB-57	Palakkad	Shoot	NA	Bacterium
58.	EB-58	Pathanamthitta	Root	NA	Bacterium
59.	EB-59	Pathanamthitta	Root	NA	Bacterium
60.	EB-60	Pathanamthitta	Pod	NA	Bacterium
61.	EB-61	Pathanamthitta	Leaf	NA	Bacterium
62.	EB-62	Pathanamthitta	Root	NA	Bacterium
63.	EB-63	Pathanamthitta	Leaf	GYEPA	Yeast
64.	EB-64	Pathanamthitta	Pod	NA	Bacterium
65.	EB-65	Pathanamthitta	Pod	KBA	Fluorescent Pseudomonad
66.	EB-66	Pathanamthitta	Root	NA	Bacterium
67.	EB-67	Pathanamthitta	Root	KBA	Fluorescent Pseudomonad
68.	EB-68	Pathanamthitta	Root	NA	Bacterium
69.	EB-69	Pathanamthitta	Root	NA	Bacterium
70.	EB-70	Pathanamthitta	Root	KBA	Fluorescent Pseudomonad
71.	EB-71	Pathanamthitta	Root	KBA	Fluorescent Pseudomonad
72.	EF-72	Pathanamthitta	Root	MRBSA****	Fungus
73.	EB-73	Palakkad	Shoot	KBA	Fluorescent Pseudomonad
74.	EB-74	Pathanamthitta	Pod	KBA	Fluorescent Pseudomonad
75.	EB-75	Pathanamthitta	Leaf	KBA	Fluorescent Pseudomonad
76.	EB-76	Pathanamthitta	Pod	GYEPA	Yeast
77.	EB-77	Palakkad	Leaf	NA	Bacterium
78.	EF-78	Palakkad	Leaf	MRBSA	Fungus
79.	EB-79	Palakkad	Shoot	KBA	Fluorescent Pseudomonad
80.	EF-80	Palakkad	Root	MRBSA	Fungus
81.	EF-81	Palakkad	Leaf	MRBSA	Fungus
82.	EB-82	Palakkad	Leaf	KBA	Fluorescent Pseudomonad

*King's B Agar, **Nutrient-Agar, *** Glucose Yeast Extract Peptone Agar, ****Martin's Rose Bengal Streptomycin Agar, EB- Endophytic bacterium, EF- Endophytic fungus

Table 4.6 *In vitro* antagonistic effect of endophytes on the pathogen

Sl. No.	Isolate	Per cent inhibition*	Sl. No.	Isolate	Per cent inhibition*
1.	EB-1	82.1	42.	EB-42	4.5
2.	EB-2	82.2	43.	EB-43	28.7
3.	EB-3	64.2	44.	EB-44	34.6
4.	EB-4	70.8	45.	EB-45	8.7
5.	EB-5	77.7	46.	EB-46	39.4
6.	EB-6	69.0	47.	EB-47	7.2
7.	EB-7	73.9	48.	EB-48	5.3
8.	EB-8	38.7	49.	EB-49	3.3
9.	EB-9	32.5	50.	EB-50	36.9
10.	EB-10	71.0	51.	EB-51	7.7
11.	EB-11	14.0	52.	EB-52	77.7
12.	EB-12	51.0	53.	EB-53	84.0
13.	EB-13	58.5	54.	EB-54	18.0
14.	EB-14	83.1	55.	EB-55	6.3
15.	EB-15	76.6	56.	EB-56	31.2
16.	EB-16	43.1	57.	EB-57	5.8
17.	EB-17	62.0	58.	EB-58	7.2
18.	EB-18	7.2	59.	EB-59	9.3
19.	EB-19	72.6	60.	EB-60	78.2
20.	EB-20	77.2	61.	EB-61	79.3
21.	EB-21	3.3	62.	EB-62	76.6
22.	EB-22	74.7	63.	EB-63	6.3
23.	EB-23	5.3	64.	EB-64	56.3
24.	EB-24	35.1	65.	EB-65	65.9
25.	EB-25	70.4	66.	EB-66	31.0
26.	EB-26	8.3	67.	EB-67	57.8
27.	EB-27	54.1	68.	EB-68	31.0
28.	EB-28	7.4	69.	EB-69	5.8
29.	EB-29	60.6	70.	EB-70	3.4
30.	EB-30	59.9	71.	EB-71	32.0
31.	EB-31	82.2	72.	EF-72	40.3
32.	EB-32	47.0	73.	EB-73	31.0
33.	EB-33	47.0	74.	EB-74	28.2
34.	EB-34	44.2	75.	EB-75	33.0
35.	EB-35	78.5	76.	EB-76	11.0
36.	EB-36	50.2	77.	EB-77	7.3
37.	EB-37	49.4	78.	EF-78	72.0
38.	EB-38	54.8	79.	EB-79	33.0
39.	EB-39	4.3	80.	EF-80	65.0
40.	EB-40	72.7	81.	EF-81	75.1
41.	EB-41	69.1	82.	EB-82	73.1

*Average of three replications, EB-Endophytic bacterium, EF- Endophytic fungus

Forty-four isolates, which registered more than 40 per cent inhibition of the pathogen were selected for screening on detached pods. Among these selected isolates, more than 75 per cent inhibition of the pathogen was noticed in the case of 14, and these include eight bacteria, five fluorescent pseudomonads and one fungus.

4.4 EFFECT OF THE ANTAGONISTIC ENDOPHYTIC ISOLATES IN REDUCING DISEASE ON DETACHED COCOA PODS AND LEAVES

Forty-four isolates of antagonistic endophytes, which showed more than 40 per cent *in vitro* inhibitory effect against the pathogen, were further evaluated for their ability in checking the disease on detached cocoa pods (Plate 4.5) as described in Materials and Methods. The endophytes tested in this study include isolates of 18 bacteria 22 fluorescent pseudomonads, and four fungi. From these, 25 efficient antagonistic isolates were selected which were further tested on detached leaves.

4.4.1 Effect of endophytic antagonists against the pathogen on detached cocoa pods

From the data on the per cent pod area infected, it was observed that, infection was more when inoculated with injury than without injury (Plate 4.6). However, less infection was noticed on the pods treated with endophytes compared to control in both the cases (Table 4.7). On pods inoculated with injury, the per cent pod area infection varied from 15.6 to 100 per cent. Pods treated with the isolate EF-78 recorded the maximum per cent reduction in pod area infection (84.4), followed by EB-31 (76.4), EF-81 (71.5), EB-35 (71.0) and EB-22 (70.1). Twelve isolates brought about 60-70 per cent reduction in infection, and sixteen isolates gave 50-60 per cent reduction. Of the remaining eleven isolates, ten reduced the per cent infection by 20-50 per cent while isolate EB-30 had no effect in reducing the infection.

When inoculated without injury, the per cent pod area infection was only 75.8 in the control after four days of inoculation. Two isolates namely EF-78 and EF-80 completely checked the infection on inoculation without injury. Four isolates *viz.*, EF-78, EF-80, EF-72 and EB-60 recorded more than 70 per cent reduction in infection when

inoculated on intact pod surface. Seventeen isolates recorded 50-70 per cent reduction in infection and twenty isolates gave 30-50 per cent, reduction. Less than 30 per cent reduction was noticed in the case of two isolates namely EB-4 and EB-16. Here also, EB-30 recorded no reduction in infection.

Based on the results as shown in Table 4.7 the forty-four antagonistic endophytes were short listed to 25, which recorded 55 per cent or more reduction in infection on pods when inoculated after giving pinpricks. These included endophytic isolates of nine bacteria, 12 fluorescent pseudomonads and four fungi.

4.4.2 Effect of endophytic antagonists against the pathogen on detached cocoa leaves

Twenty-five endophytic isolates, which showed more than 55 per cent effect in reducing the infection on cocoa pods, were further screened on detached leaves (Plate 4.7). Data on the per cent leaf area infection when inoculated with or without injury by pathogen are given in Table 4.8. Here also, the per cent leaf area infection was more when inoculated with injury than that without injury.

When inoculated after giving pinpricks, the per cent leaf area infection varied from 0.8 (EB-22) per cent to 73.6 per cent (Control). More than 70 per cent reduction in infection was recorded by all the isolates studied. The highest per cent reduction of leaf area infection over control was recorded by EB-22 (98.9) followed by EB-31 (97.6). The lowest reduction was in the case of EB-15 (73.4 per cent). The per cent infection on leaves without injury varied from zero to 15.7 (control). Five isolates namely, EB-19, EB-22, EB-31, EB-65 and EF-81 recorded no infection (Plate 4.7). Nine isolates recorded more than 90 per cent reduction in infection. Of the 25 isolates, 19 gave more than 80 per cent reduction in the disease while the remaining six gave less than 60 per cent reduction.

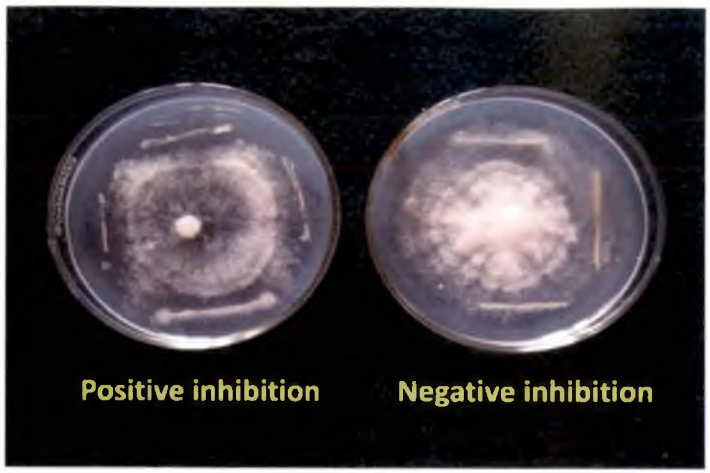

Plate 4.3 Preliminary screening of endophytes against the pathogen

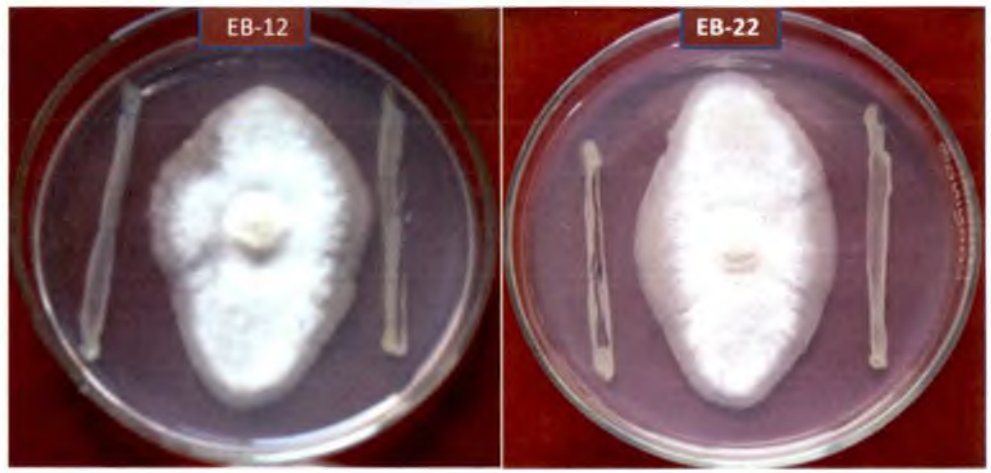

Plate 4.4 *In vitro* evaluation of endophytes in dual culture

Plate 4.5 Screening of endophytes on detached cocoa pods

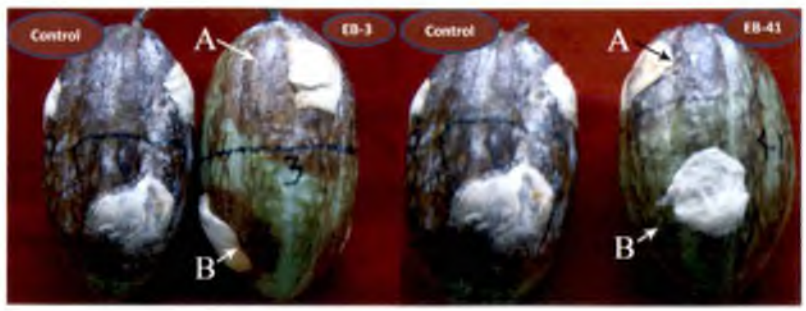

Plate 4.6 Reduction in pod infection by endophytes
A-Inoculated with injury ; B- Inoculated without injury

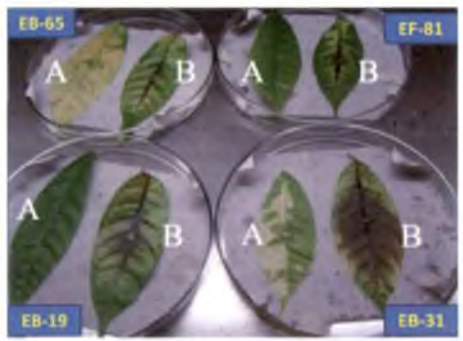

Plate 4.7 Screening of endophytes on detached cocoa leaves
A-Treated with endophytic isolate
B- Control

Table 4.7 Effect of endophytic antagonists against the pathogen on detached cocoa pods

Sl. No.	Isolate	With injury		Without injury		Sl. No.	Isolate	With injury		Without injury.	
		Per cent* pod area infection	Per cent reduction over control	Per cent pod area infection	Per cent reduction over control			Per cent pod area infection	Per cent reduction over control	Per cent pod area infection	Per cent reduction over control
1.	EB-1	44.5	55.5	15.4	60.4	23.	EB-32	73.7	26.3	34.8	41.0
2.	EB-2	47.0	53.0	6.2	69.6	24.	EB-33	46.7	53.3	38.7	37.1
3.	EB-3	54.6	45.4	45.2	30.6	25.	EB-34	48.2	51.8	38.0	37.8
4.	EB-4	65.8	34.2	56.2	19.6	26.	EB-35	20.0	71.0	25.3	50.3
5.	EB-5	40.0	60.0	27.8	48.0	27.	EB-36	48.8	51.2	43.4	32.4
6.	EB-6	39.2	60.8	30.9	44.9	28.	EB-37	54.2	45.8	43.2	32.6
7.	EB-7	50.2	49.8	40.3	35.5	29.	EB-38	45.0	55.0	31.9	43.9
8.	EB-10	75.8	24.2	38.5	37.3	30.	EB-40	38.8	61.2	16.0	59.8
9.	EB-12	51.9	48.1	35.8	40.0	31.	EB-41	34.6	65.4	10.8	65.0
10.	EB-13	48.1	51.9	36.0	39.8	32.	EB-52	33.3	66.7	9.6	66.2
11.	EB-14	48.4	51.6	37.5	38.3	33.	EB-53	43.4	56.6	16.2	59.6
12.	EB-15	40.0	60.0	34.7	41.1	34.	EB-60	31.4	68.6	1.5	74.3
13.	EB-16	47.5	52.5	46.6	29.2	35.	EB-61	49.9	57.1	13.1	62.7
14.	EB-17	53.1	46.9	41.0	34.8	36.	EB-62	43.9	56.1	19.3	56.5
15.	EB-19	32.2	67.8	25.7	50.1	37.	EB-64	35.7	64.3	22.5	53.3
16.	EB-20	45.0	55.0	30.9	44.9	38.	EB-65	35.4	64.6	24.8	51.0
17.	EB-22	29.9	70.1	11.4	64.4	39.	EB-67	32.1	67.9	8.6	67.2
18.	EB-25	43.5	56.5	30.9	44.9	40.	EF-72	33.2	66.8	3.9	71.9
19.	EB-27	51.5	48.5	36.8	39.0	41.	EF-78	15.6	84.4	0.0	75.8
20.	EB-29	61.4	38.6	38.5	37.3	42.	EF-80	43.9	56.1	0.0	75.8
21.	EB-30	100.0	0	75.8	0	43.	EF-81	28.5	71.5	22.1	53.7
22.	EB-31	23.6	76.4	21.4	51.4	44.	EB-82	48.5	51.5	10.8	65.0
						45.	Control	100.0	--	75.8	--

*Average of three replications; EB-Endophytic bacterium, EF-Endophytic fungus

Table 4.8 Effect of endophytic antagonists against the pathogen on detached cocoa leaves

Sl. No.	Isolate	With injury		With out injury		Sl. No.	Isolate	With injury		With out injury	
		Per cent leaf area infection*	Per cent reduction over control	Per cent leaf area infection	Per cent reduction over control			Per cent leaf area infection	Per cent reduction over control	Per cent leaf area infection	Per cent reduction over control
1.	EB-1	12.6	82.9	8.9	43.3	14.	EB-52	14.5	80.3	1.6	89.8
2.	EB-5	14.6	88.2	9.7	38.2	15.	EB-53	13.1	82.2	0.8	92.4
3.	EB-6	15.3	79.2	11.0	29.9	16.	EB-60	12.5	83.2	0.9	94.3
4.	EB-15	19.6	73.4	11.3	28.0	17.	EB-61	16.2	77.9	0.7	95.5
5.	EB-19	7.7	89.5	0	100.0	18.	EB-62	12.6	82.9	7.1	54.8
6.	EB-20	13.1	82.2	3.1	80.9	19.	EB-64	13.1	82.2	0.8	96.8
7.	EB-22	0.8	98.9	0	100.0	20.	EB-65	3.5	95.2	0	100.0
8.	EB-25	11.4	84.5	1.2	92.4	21.	EB-67	13.1	82.2	0.6	96.2
9.	EB-31	1.8	97.6	0	100.0	22.	EF-72	8.3	88.7	1.8	88.5
10.	EB-35	6.3	91.4	0.4	97.5	23.	EF-78	13.5	81.7	2.0	87.3
11.	EB-38	12.8	83.3	7.1	54.8	24.	EF-80	15.1	79.5	2.1	86.6
12.	EB-40	7.9	89.3	0.8	94.9	25.	EF-81	7.9	89.3	0	100.0
13.	EB-41	12.6	82.9	0.2	98.7	26.	Control	73.6	--	15.7	--

*Average of three replications; EB-Endophytic bacterium, EF-Endophytic fungus

Since all the isolates tested on detached leaves reduced the infection by more than 70 per cent when inoculated with pinpricks, all the 25 isolates were selected for evaluation of growth promoting ability.

4.5 EVALUATION OF ANTAGONISTIC ENDOPHYTES FOR GROWTH PROMOTION IN COCOA

The 25 endophytic isolates selected based on 4.3 and 4.4 were evaluated for their growth promoting ability in cocoa. These included eight isolates from Thrissur, three isolates from Idukki, two from Kottayam, six from Palakkad and six from Pathanamthitta.

A pot culture experiment was conducted as described in 3.5. Data on germination per cent are presented separately for experiments conducted using sterilized and non sterilized potting mixture (Table 4.9 and 4.10) and that on biometric observations from the four experiments were pooled and statistically analysed.

4.5.1 Effect of endophytes on germination of cocoa beans in sterilized potting mixture

Treatment with the endophytes and sowing in sterile potting mixture had an effect on earliness of germination of cocoa beans. From the data presented in Table 4.9, it is evident that seeds treated with 15 endophytic isolates showed germination on the eighth day. Twenty five per cent of the beans treated with EB-22, followed by 20 per cent of those with EB-19, EB-31, EB-35 and EF-72, germinated on the eighth day after sowing. Seven isolates effected 15 per cent germination whereas, only five per cent germination was recorded with EB-1.

On the tenth day after sowing the germination percentage of cocoa beans varied from 25 (EB-67) to 90 (EB-22 and EF-72). Six endophytes *viz.*, EB-19, EB-25, EB-31, EB-52, EF-80 and EF-81 recorded 80 per cent germination on the tenth day. Fifty to 75 per cent germination was noticed in the case of 12 isolates whereas less than 50 per cent

Table 4.9 Effiect of antagonistic endophytes on germination of cocoa beans in sterilized potting mixture

Sl. No.	Isolate	No. of cocoa beans germinated and per cent germination in sterilized potting mixture							
		8 DAS		10 DAS		12 DAS		14 DAS	
		No. of beans	Per cent germination	No. of beans	Per cent germination	No. of beans	Per cent germination	No. of beans	Per cent germination
1.	EB-1	1	5	6	30	17	85	20	100
2.	EB-5	0	0	11	55	18	90	20	100
3.	EB-6	0	0	9	45	17	85	20	100
4.	EB-15	0	0	7	35	15	75	19	95
5.	EB-19	4	20	16	80	19	95	20	100
6.	EB-20	0	0	10	50	16	80	20	100
7.	EB-22	5	25	18	90	19	95	20	100
8.	EB-25	0	0	16	80	16	80	20	100
9.	EB-31	4	20	16	80	18	90	20	100
10.	EB-35	4	20	15	75	20	100	20	100
11.	EB-38	0	0	13	65	19	95	20	100
12.	EB-40	3	15	14	70	19	95	20	100
13.	EB-41	3	15	12	60	17	85	20	100
14.	EB-52	0	0	16	80	17	85	19	95
15.	EB-53	0	0	9	45	12	60	18	90
16.	EB-60	3	15	13	65	19	95	19	95
17.	EB-61	3	15	14	70	18	90	19	95
18.	EB-62	0	0	12	60	17	85	20	100
19.	EB-64	2	10	10	50	16	80	16	80
20.	EB-65	3	15	14	70	19	95	20	100
21.	EB-67	0	0	5	25	11	55	16	80
22.	EF-72	4	20	18	90	19	95	19	95
23.	EF-78	2	10	13	65	16	80	16	80
24.	EF-80	3	15	16	80	19	95	20	100
25.	EF-81	3	15	16	80	19	95	20	100
26.	Control	0	0	9	45	13	65	17	85

DAS-Days after sowing, EB-Endophytic bacterium, EF-Endophytic fungus

was recorded by four isolates and control. Germination percentage of cocoa beans varied on 12th day after sowing also with cent per cent germination of beans treated with EB-35 and 95 per cent germination with nine other isolates. Twelve isolates recorded 80-90 per cent germination on the same day. The lowest per cent germination was noticed with EB-67 (55 per cent).

At 14 days after sowing, the germination per cent was 100 in the case of beans treated with 16 isolates, and 80-95 per cent germination in the case of the rest. The minimum germination percentage was noticed in EF-78, EB-67 and EB-64.

4.5.2 Effect of endophytes on germination of cocoa beans in non sterilized potting mixture

Data presented in Table 4.10 shows the germination of cocoa beans treated with selected endophytes in non sterile potting mixture. From the data it was evident that, treatment with endophytes resulted in better germination in non-sterile potting mixture also, but the effect was less pronounced when compared to sterile condition. The germination percentage varied from zero to 15 per cent (EB-65) at eight days after sowing. Ten per cent of the beans germinated in EB-31, EB-35, EB-40 and EB-41 and five per cent in EF-81.

The per cent germination of beans varied from zero (control) to 60 (EB-1, EB-22 and EB-38) on the tenth day with 50 to 60 per cent germination recorded by ten isolates. Twelve isolates recorded 30-50 per cent germination whereas 25 per cent germination was noticed in EB-53 and EB-64. The lowest germination percentage among the isolates tested was with EB-6 (10 per cent).

Ninety five per cent of the beans germinated on treatment with four isolates *viz.*, EB-20, EB-22, EB-35 and EB-40 at 12 days after sowing followed by 90 per cent germination in EB-1, EB-25, EB-61 and EB-67. Seventy to 95 per cent germination was

Table 4.10 Effiect of antagonistic endophytes on germination of cocoa beans in non sterilized potting mixture

Sl. No.	Isolate	No. of cocoa beans germinated and per cent germination in non-sterilized potting mixture							
		8 DAS		10 DAS		12 DAS		14 DAS	
		No. of beans	Per cent germination	No. of beans	Per cent germination	No. of beans	Per cent germination	No. of beans	Per cent germination
1.	EB-1	0	0	12	60	18	90	19	95
2.	EB-5	0	0	11	55	17	85	20	100
3.	EB-6	0	0	5	10	14	70	17	85
4.	EB-15	0	0	6	30	17	85	17	85
5.	EB-19	0	0	8	40	17	85	20	100
6.	EB-20	0	0	9	45	19	95	19	95
7.	EB-22	0	0	12	60	19	95	20	100
8.	EB-25	0	0	11	55	18	90	19	95
9.	EB-31	2	10	6	30	16	80	20	100
10.	EB-35	2	10	7	35	19	95	20	100
11.	EB-38	0	0	12	60	14	70	17	85
12.	EB-40	2	10	10	50	19	95	20	100
13.	EB-41	2	10	10	50	17	85	20	100
14.	EB-52	0	0	10	50	15	75	19	95
15.	EB-53	0	0	5	25	17	85	18	90
16.	EB-60	0	0	7	35	17	85	18	90
17.	EB-61	0	0	9	45	18	90	19	95
18.	EB-62	0	0	10	50	17	85	17	85
19.	EB-64	0	0	5	25	16	80	17	85
20.	EB-65	3	15	8	40	15	65	20	100
21.	EB-67	0	0	7	35	18	90	18	90
22.	EF-72	0	0	6	30	17	85	18	90
23.	EF-78	0	0	7	35	17	85	19	95
24.	EF-80	0	0	7	35	15	75	18	90
25.	EF-81	1	5	10	50	16	80	20	100
26.	Control	0	0	0	0	13	65	17	85

DAS-Days after sowing, EB-Endophytic bacterium, EF-Endophytic fungus

observed in all the treatments except EB-65 and control, which recorded only 65 per cent germination. After 14 days of sowing, nine isolates recorded 100 per cent germination. They are EB-5, EB-19, EB-22, EB-31, EB-35, EB-40, EB-41, EB-65 and EF-81. In all other treatments 85 to 95 per cent germination of beans was observed at 14 days after sowing.

4.5.3 Effect of endophytes on height of cocoa seedlings

Observations on height of seedlings were recorded at monthly intervals up to five months of sowing (Table 4.11). There was significant difference among the treatments with regard to the height of seedlings at each stage of observation. One month after sowing, plants in treatment T_{10} (EB-35) recorded the maximum height of seedlings (16.5 cm), followed by T_9 (EB-31). These two were on par with 18 other treatments. The minimum height was recorded in T_{17} (EB-61) and was on par with 17 other treatments including control (T_{26}). At two months after sowing, also T_{10} (EB-35) ranked first in seedling height followed by T_9 (EB-31) however, these were on par with plants in all other treatments except T_{17} (EB-61) which had the minimum height.

After three months of sowing, seedlings in treatment T_9 (EB-31) and T_{10} (EB-35) recorded equal and the maximum height. At this stage the minimum height was recorded for seedlings in T_{26} (control). At four months after sowing, T_9 (EB-31) showed the highest seedling height (39.8cm) followed by T_{10} (EB-35) (39.4cm) and were on par with 16 other treatments. Nevertheless, they are superior to eight other treatments including control (T_{26}). However, seedlings in T_{18} recorded the least height.

After five months also, plants in treatments T_9 (44.6 cm) had the maximum seedling height closely followed by T_{10} (44.4 cm), T_{12} (EB-40), T_{20} (EB-65), T_{25} (EB-81), T_5 (EB-19), T_7 (EB-22), T_{13} (EB-41),T_4 (EB-15) and T_{21} (EB-67). These treatments were on par with other 11 treatments. T_{18} (EB-62) recorded the lowest height which was significantly lower to control (T_{26}).

Table 4.11 Effect of the antagonistic endophytes on height of cocoa seedlings

Sl. No.	Isolate	Seedling height* (cm)					
		1MAS	2MAS	3MAS	4MAS	5MAS	Per cent +/- over control (5MAS)
1.	EB-1	13.7 abcdef	20.6 ab	29.2 abc	37.4 abcde	40.4 abcd	+6.4
2.	EB-5	14.0 abcdef	19.0 ab	27.2 cde	33.2 defg	36.6 bcd	+17.4
3.	EB-6	14.4 abcdef	21.2 ab	29.4 abc	34.4 abcdef	39.4 abcd	+14.5
4.	EB-15	13.7 abcdef	20.5 ab	28.6 abcde	34.0 cdefg	42.2 ab	+22.7
5.	EB-19	15.2 abcde	22.1 a	29.8 abc	38.2 abcd	43.0 ab	+25.0
6.	EB-20	12.4 ef	21.0 ab	29.2 abc	37.0 abcde	41.6 abc	+20.9
7.	EB-22	15.2 abcde	22.2 a	30.2 abc	38.0 abcd	42.8 ab	+24.4
8.	EB-25	13.1 cdef	21.0 ab	29.4 abc	36.2 abcde	39.4 abcd	+14.5
9.	EB-31	16.0 ab	23.3 a	33.0 a	39.8 a	44.6 a	+29.7
10.	EB-35	16.5 a	23.6 a	33.0 a	39.4 ab	44.4 a	+29.1
11.	EB-38	14.4 abcdef	21.3 ab	29.6 abc	34.6 abcdef	41.0 abc	+19.2
12.	EB-40	15.6 abc	23.0 a	32.6 ab	38.8 abc	44.4 a	+29.1
13.	EB-41	15.2 abcde	22.7 a	30.8 abc	37.8 abcd	42.2 ab	+22.7
14.	EB-52	14.9 abcdef	20.9 ab	27.6 cde	29.6 gh	39.8 abcd	+15.7
15.	EB-53	12.7 def	21.6 a	27.8 bcde	36.0 abcde	40.6 abcd	+18.0
16.	EB-60	14.6 abcdef	21.9 a	26.2 cde	34.2 abcdef	41.2 abc	+19.8
17.	EB-61	12.3 f	16.5 b	29.0 abc	37.4 abcde	39.4 abcd	+14.5
18.	EB-62	14.0 abcdef	21.7 a	28.6 abcde	26.4 h	29.0 e	-15.7
19.	EB-64	15.0 abcdef	21.6 a	29.8 abc	33.4 defg	40.8 abc	+18.6
20.	EB-65	15.6 abc	22.8 a	31.0 abc	38.8 abc	44.2 a	+28.5
21.	EB-67	13.7 abcdef	21.2 ab	28.8 abcd	32.6 efg	42.0 ab	+22.1
22.	EF-72	14.9 abcdef	20.6 ab	29.4 abc	34.8 abcdef	35.2 cd	+2.3
23.	EF-78	13.6 bcdef	21.2 ab	24.4 de	33.4 defg	38.6 abcd	+12.2
24.	EF-80	14.8 abcdef	21.8 a	27.8 abcde	34.2 abcdef	36.8 bcd	+7.0
25.	EF-81	15.6 abc	22.8 a	31.0 abc	38.6 abc	43.6 a	+26.7
26.	Control	13.3 bcdef	19.0 ab	24.2 e	31.0 fg	34.4 d	--

* Pooled mean of four experiments, Values followed by same super script are not significantly different, MAS-Months after sowing, EB-Endophytic bacterium, EF-Endophytic fungus

The efficiency of selected antagonistic endophytes in increasing the height of cocoa seedlings compared to control was worked out after five months of sowing. Eleven endophytes exerted more than 20 per cent efficiency in augmenting seedling height. Among them, T_9 (EB-31), T_{10} (EB-35), T_7 (EB-22) and T_{20} (EB-65) recorded more than 28 per cent increase compared to untreated plants. However, treatment T_{18} (EB-62) had a negative effect on seedling height. T_{22} (EF-72), T_1 (EB-1) and T_{24} (EF-80) showed less than 10 per cent efficiency in increasing the height.

4.5.4 Effect of endophytes on number of leaves of cocoa seedlings

Number of leaves of the seedlings was recorded at monthly interval up to five months of sowing. Data presented in Table 4.12 reveals that the treatments differ significantly with regard to number of leaves at each stage of observation.

During the experiment, the maximum number of leaves was recorded by T_9 (EB-31) at all the stages of observation. At one month after sowing T_9 (EB-31) was closely followed by T_{10} (EB-35), T_{12} (EB-40) and T_{20} (EB-65) whereas, the minimum number of leaves was in T_{18} (EB-62). Plants treated with T_9 (EB-31) and T_{10} (EB-35) had the maximum number of leaves at two months after sowing which were followed by T_{20} (EB-65), T_{13} (EB-40), T_7 (EB-22), T_5 (EB-19), and T_{25} (EF-81). These were on par with 18 other treatments. T_{18} (EB-62) recorded the minimum number of leaves at two MAS also. Again at three months of sowing, T_9 (EB-31), T_{10} (EB-35),T_{12} (EB-40) and T_{13}(EB-41) were having the maximum number of leaves and these were on par with all other treatments except T_{26} (control). Maximum number of leaves at four months was also in T_9 (EB-31) followed by T_{10} (EB-35). However, these were on par with each other and with 14 others. Lowest number of leaves was present in T_{26} (control) which was also on par with 21 other treatments.

At five months after sowing plants in T_9 (EB-31) recorded the highest number of leaves followed by T_{10} (EB-35). These two treatments were superior to others. Next highest number of leaves were for T_{12} (EB-40) and T_{20} (EB-65). Nevertheless, these were on par with all the other treatments except T_9(EB-31) and T_{10}(EB-35). The least

Table 4.12 Effect of the antagonistic endophytes on number of leaves of cocoa seedlings

Sl. No.	Isolate	Number of leaves of cocoa seedlings*					
		1MAS	2MAS	3MAS	4MAS	5MAS	Per cent +/- over control (5MAS)
1.	EB-1	2.6 abc	5.8 abc	9.6 ab	14.0 d	15.0 c	-12.8
2.	EB-5	2.0 bc	5.8 abc	10.0 ab	15.6 abcd	17.8 bc	+3.5
3.	EB-6	2.8 abc	5.8 abc	11.2 a	16.0 abcd	19.6 bc	+14.0
4.	EB-15	2.8 abc	5.8 abc	10.4 ab	15.0 bcd	18.0 bc	+4.7
5.	EB-19	3.0 abc	6.4 abc	11.6 a	16.4 abcd	20.6 bc	+19.8
6.	EB-20	2.8 abc	5.8 abc	9.8 ab	16.0 abcd	17.4 bc	+1.2
7.	EB-22	3.0 abc	6.4 abc	11.6 a	16.4 abcd	20.6 bc	+19.8
8.	EB-25	2.2 bc	5.0 abc	10.0 ab	15.0 bcd	19.0 bc	+10.5
9.	EB-31	3.8 a	6.8 a	12.0 a	18.4 a	28.2 a	+64.0
10.	EB-35	3.4 ab	6.8 a	12.0 a	17.8 ab	27.8 a	+61.6
11.	EB-38	2.8 abc	5.0 abc	10.4 ab	16.2 abcd	17.0 bc	-1.1
12.	EB-40	3.4 ab	6.6 ab	12.0 a	17.2 abc	21.8 b	+26.7
13.	EB-41	3.0 abc	6.4 abc	12.0 a	16.4 abcd	20.6 bc	+19.8
14.	EB-52	2.8 abc	5.2 abc	10.8 a	16.0 abcd	19.0 bc	+10.5
15.	EB-53	2.0 bc	5.6 abc	10.2 ab	14.6 cd	19.8 bc	+15.1
16.	EB-60	2.8 abc	5.8 abc	10.4 ab	16.2 abcd	18.6 bc	+8.1
17.	EB-61	2.4 abc	6.2 abc	10.8 a	14.8 cd	18.6 bc	+8.1
18.	EB-62	1.6 d	4.6 c	8.8 ab	13.8 d	17.0 bc	-1.1
19.	EB-64	2.8 abc	6.0 abc	9.8 ab	14.8 cd	19.6 bc	+14.0
20.	EB-65	3.2 ab	6.6 ab	11.8 a	17.2 abc	21.8 b	+26.7
21.	EB-67	2.4 abc	6.2 abc	10.2 ab	13.8 d	19.2 bc	+11.8
22.	EF-72	2.6 abc	4.8 bc	10.2 ab	16.2 abcd	17.8 bc	+3.5
23.	EF-78	2.8 abc	5.8 abc	9.8 ab	16.0 abcd	20.4 bc	+18.6
24.	EF-80	2.4 abc	5.6 abc	10.0 ab	15.0 cd	15.0 c	-12.8
25.	EF-81	3.0 abc	6.4 abc	11.4 a	16.4 abcd	20.8 b	+20.9
26.	Control	2.2 bc	5.6 abc	7.0 b	13.6 d	17.2 bc	--

* Pooled mean of four experiments, Values followed by same super script are not significantly different, MAS-Months after sowing, EB-Endophytic bacterium, EF-Endophytic fungus

Table 4.13 Effect of the antagonistic endophytes on girth at collar and fresh and dry weight of shoot of cocoa seedlings

Sl. No.	Isolate	Girth at collar (cm.)*	Per cent +/- over control	Shoot fresh weight. (g.)*	Per cent +/- over control	Shoot dry weight. (g.)*	Per cent +/- over control
1.	EB-1	2.2^{bc}	-12.0	27.1^{abc}	- 2.5	11.1^{abc}	+9.9
2.	EB-5	3.1^{abc}	+24.0	22.6^{bc}	-18.7	8.2^{c}	-18.8
3.	EB-6	3.2^{abc}	+28.0	23. 6^{abc}	-15.1	8.9^{bc}	-11.8
4.	EB-15	2.7^{bc}	+8.0	25. 8^{abc}	- 7.2	9.9^{abc}	-2.0
5.	EB-19	3.2^{abc}	+28.0	32.1^{abc}	+4.3	12.1^{abc}	+19.8
6.	EB-20	3.0^{abc}	+20.0	25.9^{abc}	-6.8	10.4^{abc}	+2.9
7.	EB-22	3.2^{ab}	+28.0	30.0^{abc}	+7.9	11.8^{abc}	+16.8
8.	EB-25	3.0^{abc}	+20.0	30.2^{abc}	+8.6	11.7^{abc}	+15.8
9.	EB-31	3.6^{a}	+44.0	34.6^{a}	+24.5	14.0^{a}	+38.6
10.	EB-35	3.4^{ab}	+36.0	34.7^{a}	+24.8	14.2^{a}	+40.6
11.	EB-38	3.1^{abc}	+24.0	28.1^{abc}	+1.1	11.5^{abc}	+13.9
12.	EB-40	3.3^{ab}	+32.0	34.4^{ab}	+23.7	13.3^{ab}	+31.7
13.	EB-41	3.2^{abc}	+28.0	30.5^{abc}	+9.7	11.8^{abc}	+16.8
14.	EB-52	3.0^{abc}	+20.0	27.1^{abc}	-2.5	11.1^{abc}	+9.9
15.	EB-53	2.9^{abc}	+16.0	22.5^{c}	-19.1	8.1^{c}	-19.8
16.	EB-60	2.9^{abc}	+16.0	26.4^{abc}	- 5.0	10.6^{abc}	+5.0
17.	EB-61	3.1^{abc}	+24.0	27.3^{abc}	-1.8	11.5^{abc}	+13.9
18.	EB-62	2.9^{abc}	+16.0	25.1^{abc}	-9.7	10.2^{abc}	+1.0
19.	EB-64	2.9^{abc}	+16.0	24.2^{abc}	-12.9	9.0^{bc}	-1.9
20.	EB-65	3.3^{ab}	+32.0	34.1^{abc}	+22.7	12.7^{abc}	+25.7
21.	EB-67	3.0^{abc}	+20.0	26.1^{abc}	- 6.1	10.5^{abc}	+4.0
22.	EF-72	3.1^{abc}	+24.0	26.1^{abc}	- 6.1	10.5^{abc}	+4.0
23.	EF-78	3.0^{abc}	+20.0	25.4^{abc}	- 8.6	10.2^{abc}	+1.0
24.	EF-80	3.0^{abc}	+20.0	29.0^{abc}	+4.3	11.6^{abc}	+14.9
25.	EF-81	3.3^{ab}	+32.0	32.4^{abc}	+16.5	12.5^{abc}	+23.8
26.	Control	2.5^{bc}	--	27.8^{abc}	--	10.1^{abc}	--

* Pooled mean of four experiments, Values followed by same super script are not significantly different, EB-Endophytic bacterium, EF-Endophytic fungus

number of leaves at five months was with T_1 (EB-1) and T_{24} (EF-80) which had lesser number than control. Twenty treatments had positive effect on the number of leaves at this stage. There were 64 per cent more leaves in T_9 (EB-31) compared to control, followed by T_{10} (EB-35) with 61.6 per cent. More than 20 per cent increase was effected by T_{12} (EB-40), T_{20} (EB-65) and T_{25} (EB-81). Four treatments had a negative effect on leaves, they are T_1 (EB-1), T_{11} (EB-38), T_{18} (EB-62) and T_{24} (EF-80).

4.5.5 Effect of endophytes on girth at collar

Data on girth at collar region of the seedlings at five months after sowing (Table 4.13) revealed a significant difference among the treatments. All the treatments except T_1 (EB-1) recorded a girth at collar higher than that of control. The maximum girth was for T_9 (EB-31) followed by T_{10} (EB-35) which showed 44 and 36 per cent increase over control respectively. These two treatments were on par with 20 other treatments also. Girth at collar was the minimum for T_1 (EB-1).

4.5.6 Effect of endophytes on fresh weight of shoot

Fresh weight of the shoot was recorded at five months after sowing. Data as per Table 4.13 shows significant difference in fresh weight of shoots among treatments. The maximum weight was recorded by T_{10} (EB-35) followed by T_9 (EB-31) which were on par with twenty-two other treatments including T_{26} (control). The minimum weight was recorded by T_{15} (EB-53), which was lower than the control. Eleven treatments gave increase in fresh weight of shoots compared to control with maximum increase of 24.8 per cent by T_{10} (EB-35).

4.5.7 Effect of endophytes on dry weight of shoot

Observations on dry weight of shoot at five months after sowing are also presented in Table 4.13, which showed a significant difference among treatments. T_{10} (EB-35) recorded the highest dry weight followed by T_9 (EB-31) however, these were on par with twenty other treatments. Except five, all the other treatments gave higher dry

Table 4.14 Effect of the antagonistic endophytes on root length and fresh and dry weight of root of cocoa seedlings

Sl. No.	Isolate	Root length (cm)	Per cent +/- over control	Root fresh weight* (g.)	Per cent +/- over control	Root dry weight* (g.)	Per cent +/- over control
1.	EB-1	20.5 ^{cdefg}	+2.5	7.2 ^{bcdefg}	+7.5	2.7 ^{bcdef}	0.0
2.	EB-5	20.2 ^{cdefg}	+1.0	6.4 ^{defg}	-4.5	2.4 ^{cdef}	-11.1
3.	EB-6	20.2 ^{cdefg}	+1.0	6.2 ^{efg}	-7.5	2.4 ^{cdef}	-11.1
4.	EB-15	22.1 ^{abcdefg}	+10.5	7.8 ^{abcdefg}	+16.4	2.9 ^{abcdef}	+7.4
5.	EB-19	24.5 ^{abcd}	+22.5	11.3 ^{abcde}	+68.7	3.2 ^{abcde}	+18.5
6.	EB-20	16.2 ^{g}	-19.0	10.1 ^{abcdefg}	+50.7	2.0 ^{cdef}	-3.7
7.	EB-22	24.0 ^{abcde}	+20	11.1 ^{abcdef}	+65.7	3.0 ^{abcdef}	+11.1
8.	EB-25	21.7 ^{abcdefg}	+8.5	5.3 ^{g}	-20.9	2.8 ^{abcdef}	+3.7
9.	EB-31	27.2 ^{a}	+36.0	13.3 ^{a}	+98.5	4.3 ^{a}	+59.3
10.	EB-35	27.2 ^{a}	+36.0	12.4 ^{ab}	+85.1	4.1 ^{ab}	+51.9
11.	EB-38	23.0 ^{abcde}	+15.0	9.8 ^{abcdefg}	+46.3	2.8 ^{abcdef}	+3.7
12.	EB-40	26.0 ^{abc}	+30.0	12.3 ^{ab}	+83.6	3.4 ^{abc}	+29.5
13.	EB-41	25.2 ^{abcd}	+26.0	12.0 ^{abcd}	+79.1	3.3 ^{abcd}	+22.2
14.	EB-52	22.8 ^{abcde}	+14.0	10.9 ^{abcdefg}	+62.7	2.9 ^{abcdef}	+7.4
15.	EB-53	18.2 ^{efg}	-4.0	5.3 ^{g}	-20.9	1.8 ^{ef}	-33.3
16.	EB-60	16.8 ^{fg}	-16.0	7. 8 ^{abcdefg}	+16.4	2.9 ^{abcdef}	+7.4
17.	EB-61	23.6 ^{abcde}	+18.0	7.2 ^{bcdefg}	+7.5	2.8 ^{abcdef}	+3.7
18.	EB-62	16.6 ^{fg}	-17.0	5.6 ^{fg}	+16.4	1.9 ^{def}	-29.6
19.	EB-64	22.2 ^{abcdef}	+11.0	9.1 ^{abcdefg}	+35.8	2.9 ^{abcdef}	+7.4
20.	EB-65	26.6 ^{abc}	+33.0	12.2 ^{abc}	+82.1	3.0 ^{abcdef}	+11.1
21.	EB-67	22.8 ^{abcde}	+14.0	8.4 ^{abcdefg}	+25.4	2.9 ^{abcdef}	+7.4
22.	EF-72	21.0 ^{bcdefg}	+5.0	5.8 ^{efg}	-13.4	2.3 ^{cdef}	-14.8
23.	EF-78	19.0 ^{defg}	-5.0	9.0 ^{abcdefg}	+34.3	2.9 ^{abcdef}	+7.4
24.	EF-80	23.6 ^{abcde}	+18.0	7.3 ^{bcdefg}	+89.6	2.8 ^{abcdef}	+3.7
25.	EF-81	25.4 ^{abc}	+27.0	12.1 ^{abc}	+80.6	3.0 ^{abcdef}	+11.1
26.	Control	20.0 ^{cdefg}	--	6.7 ^{cdefg}	--	2.7 ^{abcdef}	--

* Pooled mean of four experiments, Values followed by same super script are not significantly different, MAS-Months after sowing, EB-Endophytic bacterium, EF-Endophytic fungus

weight of shoot than control. The lowest weight was recorded by T_{15} (EB-53), whereas a percentage increase of 40.6 over control was recorded by T_{10} (EB-35).

4.5.7 Effect of endophytes on root length and fresh and dry weight of roots

The root length recorded at five months after sowing revealed a significant difference among the treatments (Table 4.14). The maximum root length was noticed in T_9 (EB-31) and T_{10} (EB-35) with a 36 per cent increase over control whereas, the minimum root length was observed in T_6 (EB-20). Of the 25 endophytic isolates tested, 20 had a positive effect on the root length of cocoa seedlings while, five had negative effect. Fresh and dry weight of roots at five months after sowing also showed a significant difference. Fresh weight of roots varied from 5.3 to 13.3 g and dry weight from 1.8 to 4.3 g. The maximum fresh and dry weight of roots was noticed in T_9 (EB-31) followed by T_{10} (EB-35). These two treatments were on par with 14 and 17 other treatments with regard to fresh and dry weight respectively, but these were superior to the control with regard to fresh weight. Fresh weight was the minimum in the case of T_8 (EB-25) but minimum dry weight recorded in T_{15} (EB-53).

4.5.8 Selection of potential endophytes

From the results presented in Tables 4.9 to 4.14 it is evident that, the endophytes tested differ in the growth promoting ability. Those isolates, which had maximum efficiency in augmenting various growth parameters studied, were selected for further evaluation. It was noticed that, eight isolates *viz.*, EB-19, EB-22, EB-31, EB-35, EB-40, EB-41, EB-65 and EF-81 had more than 20 per cent efficiency over control in increasing seedling height, more than 19 per cent efficiency on number of leaves, 16 per cent effect on dry weight of shoot and more than 28 per cent effect on girth at collar. They have shown more efficiency on enhancing the root parameters too. Further, from the effect on the disease on detached pods and leaves, it was observed that these isolates had more than 60 per cent efficiency in reducing the disease on detached pods (Table 4.7) and 80 per cent efficiency on detached leaves (Table 4.8) when inoculated with injury. These

Table 4.15 Potential antagonistic endophytes selected based on growth promoting and antagonistic effect

Sl. No.	Isolate	District	Part of the plant	Medium of isolation	Type of organism
1.	EB-19	Thrissur	Leaf	KBA	Fluorescent pseudomonad
2.	EB-22	Thrissur	Pod	NA	Bacterium
3.	EB-31	Thrissur	Pod	KBA	Fluorescent pseudomonad
4.	EB-35	Idukki	Pod	NA	Bacterium
5.	EB-40	Thrissur	Pod	KBA	Fluorescent pseudomonad
6.	EB-41	Idukki	Pod	KBA	Fluorescent pseudomonad
7.	EB-65	Pathanamthitta	Pod	KBA	Fluorescent pseudomonad
8.	EF-81	Palakkad	Leaf	MRBSA	Fungus

KBA-King's B Agar, NA-Nutrient Agar, MRBSA- Martin's Rose Bengal Streptomycin Agar, EB- Endophytic bacterium, EF- Endophytic fungus

were selected as potential endophytes since they exhibited good antagonism coupled with good growth promoting ability, and these include isolates of five fluorescent pseudomonads, two bacteria and one fungus. The details of the potential endophytes are presented in Table 4.15.

4.6 MECHANISMS OF ACTION OF ENDOPHYTES

The potential endophytes as per Table 4.15 were subjected to various tests for studying the mechanisms of antagonism against *Phytophthora palmivora* and growth promotion in cocoa in comparison with standard cultures of *Pseudomonas fluorescens* from KAU (Pf_1) and TNAU (Pf_2).

4.6.1 Ammonia Production

Production of ammonia by the isolates was detected by change in colour of peptone broth media on addition of Nessler's reagent. The potential isolates produced varying amounts of ammonia as evidenced by varying colour changes of the media (Table 4.16). The isolates EB-35, EB-40, EF-81 and Pf_1 produced more ammonia (Fig. 4.1) as evidenced by the colour of the medium changed to brownish orange and they were scored as four. EB-31, EB-41 and EB-65 were intermediate with regard to ammonia production since the colour change they produced was to orange on addition of Nessler's reagent, accordingly they were scored as three. Least amount of ammonia was produced by EB-22 and Pf_2 (colour of the medium changed to orange yellow) and they were scored as two. No ammonia was produced by EB-19 and it was given score one.

4.6.2 Production of HCN

The selected endophytes were tested for their ability to produce hydrogen cyanide (HCN). It was observed that none of the isolates produced hydrogen cyanide and all were therefore scored as one.

Table 4.16 Production of ammonia by potential isolates

Sl. No.	Isolate	Colour change of peptone water on addition of Nessler's reagent	Score
1.	EB-19	Yellow	1
2.	EB-22	Orange yellow	2
3.	EB-31	Orange	3
4.	EB-35	Brownish orange	4
5.	EB-40	Brownish orange	4
6.	EB-41	Orange	3
7.	EB- 65	Orange	3
8.	EF-81	Brownish orange	4
9.	Pf_1	Brownish orange	4
10.	Pf_2	Orange yellow	2
11.	Control	Yellow	1

EB-Endophytic bacterium, EF- Endophytic fungus, Pf_1- *P. fluorescens* (KAU), Pf_2- *P. fluorescens* (TNAU)

Table 4.17 Phosphate solubilization by potential isolates

Sl. No.	Isolate	*P solubilization zone (mm)	P solubilization ($mg.50ml^{-1}$)*	Score
1.	EB-19	5.3	6.1	2
2.	EB-22	9.1	7.0	2
3.	EB-31	10.2	14.3	4
4.	EB-35	9.1	15.0	4
5.	EB-40	11.3	14.0	4
6.	EB-41	9.1	4.0	1
7.	EB-65	10.2	7.0	2
8.	EF-81	0	0	1
9.	Pf_1	7.5	12.5	3
10.	Pf_2	8.7	14.1	4
11.	Control	0	0	1

* Mean of three replications, EB- Endophytic bacterium, EF-Endophytic fungus, Pf_1 -*P. fluorescens* (KAU), Pf_2-*P. fluorescens* (TNAU), Score>1<5 $mg.50ml^{-1}$ = 1; >5<10 $mg.50ml^{-1}$ = 2; >10<14 $mg.50\ ml^{-1}$ = 3 and >14 $mg.50ml^{-1}$ = 4.

4.6.3 Phosphate solubilization

The phosphorus solubilizing ability of the selected promising endophytes was tested on Pikovaskya's agar as well as in liquid medium, which contain insoluble tricalcium phosphate as sole source of P. Out of the ten isolates including the reference cultures (Pf_1 and Pf_2), nine exhibited ability to solubilize tricalcium phosphate (TCP). The clear zone of P solubilization on Pikovskya's agar ranged from 5.3 to 11.3 mm. EB-40 produced the clear zone with the maximum diameter (Table 4.17) followed by EB-65 and EB-31. Bioassay revealed that EB-35 possess more capacity to solubilize P than any other isolate tested. Out of the ten isolates tested, four, namely EB-31, EB-35, EB-40 and Pf_2 showed P solubilization of 14mg $50ml^{-1}$ or above (Fig. 4.2), and hence they were scored as four (Fig.4.2). Pf_1 released more than 10 mg $50ml^{-1}$ of soluble P it was scored three while four isolates *viz.*, EB-19, EB-22 and EB-65, released P between five and 10 mg $50ml^{-1}$ and these were scored as two. The fungal isolate EF-81 did not solubilize detectable amount of tricalsium phosphate and EB-41 exhibited a solubilization of less than five mg $50ml^{-1}$ only. Hence, these were given the score one.

4.6.4 IAA Production

All the isolates, *viz.*, eight endophytes and two reference cultures produced varying levels of IAA, ranging from 7.5 to 56.8 $\mu g\ ml^{-1}$ (Table 4.18). The maximum quantity of IAA was produced by EB-35 followed by EB-40 (Fig. 4.3). These two isolates and EB-65 produced more than 30 $\mu g.\ ml^{-1}$ of IAA (Fig. 4.3). Hence they were scored as four. EB-31 which produced IAA between 25 and 30 $\mu g\ ml^{-1}$ was given score three. Isolates which showed IAA production of 10 - 25 $\mu g.\ ml^{-1}$ (EB-22, EF-81, Pf_1, Pf_2 and EB-41) were scored as two. EB-19 with only 7.5 $\mu g.\ ml^{-1}$ (<10) IAA produced and control were scored as one.

4.6.5 Antagonism Index (AI)

The selected endophytic isolates were screened for their *in vitro* inhibitory effect against the pathogen to find out the antagonism index (AI). The selected isolates varied

Table 4.18 Production of indole acetic acid (IAA) by potential isolates

Sl. No.	Isolate	IAA produced (μg. ml^{-1})*	Score
1.	EB-19	7.5	1
2.	EB-22	20.0	2
3.	EB-31	26.0	3
4.	EB-35	56.8	4
5.	EB-40	54.0	4
6.	EB-41	11.0	2
7.	EB-65	31.5	4
8.	EF-81	16.5	2
9.	Pf_1	19.1	2
10.	Pf_2	17.0	2
11.	Control	0	1

* Mean of three replications, EB-Endophytic bacterium, EF- Endophytic fungus Pf_1- *P. fluorescens* (KAU), Pf_2- *P. fluorescens* (TNAU), Score>5<10 µg. ml^{-1} = 1; >10<25 µg. ml^{-1} = 2; >25<30 µg. ml^{-1} = 3 and >30 µg. ml^{-1} = 4

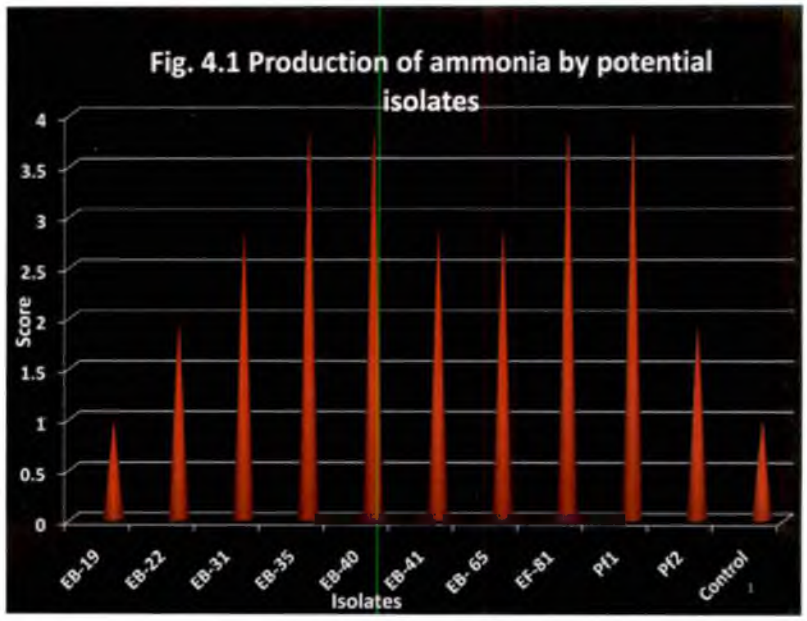
Fig. 4.1 Production of ammonia by potential isolates
Score
4
3.5
3
2.5
2
1.5
1
0.5
0
EB-19
EB-22
EB-31
EB-35
EB-40
EB-41
EB- 65
EF-81
PI1
PI2
Control
Isolates

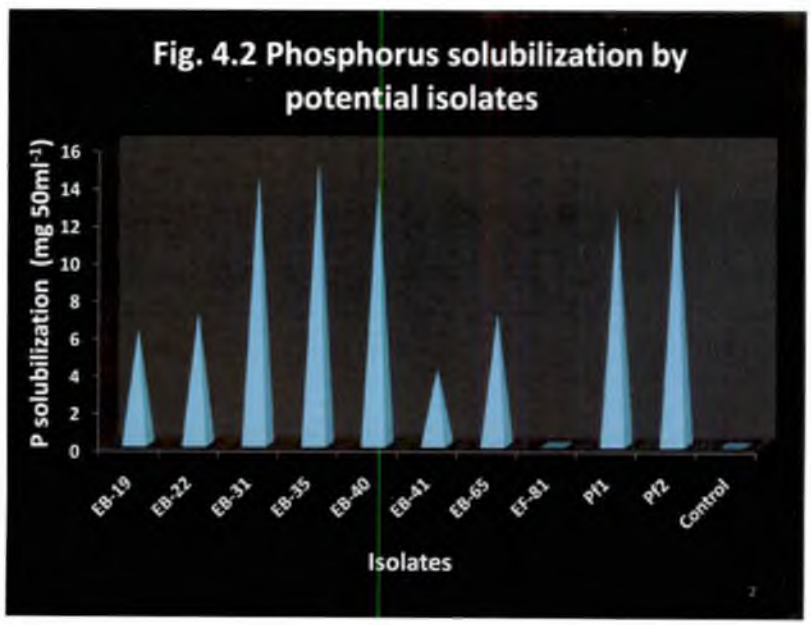
Fig. 4.2 Phosphorus solubilization by potential isolates
P solubilization (mg 50ml-1)
16
14
12
10
8
6
4
2
0
EB-19
EB-22
EB-31
EB-35
EB-40
EB-41
EB-65
EF-81
PI1
PI2
Control
Isolates

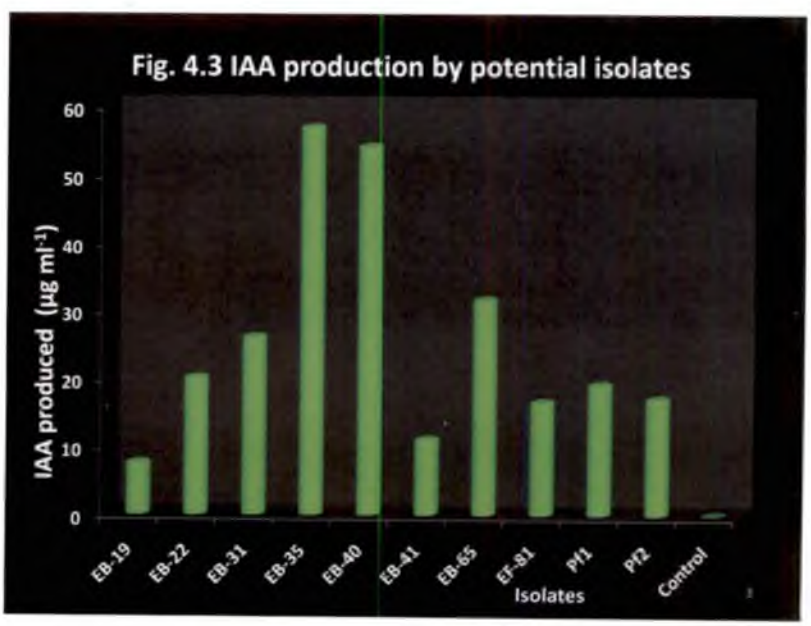
Fig. 4.3 IAA production by potential isolates
IAA produced (µg ml-1)
60
50
40
30
20
10
0
EB-19
EB-22
EB-31
EB-35
EB-40
EB-41
EB-65
EF-81
PI1
PI2
Control
Isolates

Table 4.19 Antagonism index (AI) of the potential isolates against the pathogen

Sl. No.	Isolate	Per cent inhibition	Inhibition zone (mm)	TIME (score)	CB (score)	AI	score
1.	EB-19	72.6	6.50	2	2	1888	3
2.	EB-22	74.7	6.70	2	2	2002	3
3.	EB-31	82.2	7.40	2	2	2433	4
4.	EB-35	78.5	7.07	2	2	2220	4
5.	EB-40	72.7	6.54	2	2	1902	3
6.	EB-41	69.1	6.22	2	2	1719	2
7.	EB-65	65.9	5.93	2	2	1563	2
8.	EF-81	75.1	6.76	2	2	2031	3
9.	Pf_1	76.4	6.88	2	2	2102	3
10.	Pf_2	75.0	6.75	2	2	2025	3
11.	Control	0	0	0	0	0	

EB Endophytic bacterium, EF Endophytic fungus Pf_1 *Pseudomonas fluorescens* (KAU), Pf_2 *Pseudomonas fluorescens* (TNAU), CB- Colonization behavior, TIME – Time taken by pathogen/antagonist to over grow, Score>1200<1500 = 1; >1500<1800 = 2; >1800< 2200= 3 and >2200 = 4

Table 4.20 Effect of potential isolates on the vigour index (VI) of cocoa seedlings

Sl. No.	Isolate	Mean shoot length (cm)	Mean root length (cm)	Per cent germination	VI	Score
1.	EB-19	41.9	25.4	100	67.3	3
2.	EB-22	44.8	26.0	100	70.8	4
3.	EB-31	44.8	27.2	100	72.0	4
4.	EB-35	45.7	26.6	100	72.3	4
5.	EB-40	45.1	24.5	100	69.6	4
6.	EB-41	42.1	23.6	100	65.7	3
7.	EB-65	42.8	25.2	100	68.0	4
8.	EF-81	42.9	27.2	100	70.1	4
9.	Pf_1	44.5	27.3	100	71.8	4
10.	Pf_2	41.3	20.8	100	62.1	3
11.	Control	33.3	22.0	93	51.4	--

EB- Endophytic bacterium, EF- Endophytic fungus Pf_1 – *P. fluorescens* (KAU), Pf_2 *P. fluorescens* (TNAU)
Score>44<52 = 1; >52<60 = 2; >60< 68= 3 and >68 = 4

in their inhibitory effect on the pathogen as shown in the Table 4.19 (Fig. 4.4). The antagonism index (AI) was the highest for EB-31 followed by EB-35, Pf_1, EF-81, Pf_2 and EB-22, in that order. AI recorded by EB-31 and EB-35 are above 2200 and hence scored as four. Pf_1, EF-81, Pf_2, EB-22, EB-19 and EB-40 recorded AI above 1800 and hence scored three. The rest of the isolates with AI less than 1800 were scored as two.

4.6.6 Vigour Index (VI)

Based on the mean shoot length, root length and per cent germination of cocoa seedlings treated with the selected eight endophytic isolates, vigour index (VI) of the seedlings was calculated. It was compared with that of seedlings treated with the reference cultures (Table 4.20) (Fig.4.5). The highest value for vigour index (72.3) was recorded for seedlings treated with EB-31 followed by EB-35 (72.0) and Pf_1 (71.8) respectively. All treated seedlings had a VI value higher than that of untreated seedlings. Treatment with isolates EB-31, EB-35, Pf_1, EB-22, EF-81, EB-40 and EB-65 resulted in VI values 68 or above and they are scored as four. EB-19, EB-41, and Pf_2 gave less vigour index hence scored as three.

4.6.7 PGPE Index

The PGPE index, originally developed by Samanta and Dutta (2004) for comparing Plant Growth Promoting Rhizobacteria (PGPR index) was calculated for the potential endophytes for ranking them with due consideration of all the growth promoting parameters (Table 4.21) (Fig.4.6). The index was calculated for two reference cultures also for comparison. It was found that EB-35 and EB-40 have the maximum score in most of the parameters studied so they have the highest PGPE index of 87.5 and 83.3 respectively. Six isolates out of the eight selected recorded high PGPI (more than 60). They are EB-31, EB-35, EB-40, EB-65, Pf_1 and EF-81. EB-22, and Pf_2 had PGP index between 55 and 60 hence they were assigned the score three. The isolates EB-19 and EB-41 had the index between 45 and 55 and were given score as two.

Table 4.21 PGPE Index of potential isolates

Sl. No.	Isolate	Vigour Index	AI	IAA	NH_3 production	Phosphorus solubilization	HCN	PGPE/ PGP index	Score
1.	EB-19	3	3	1	3	2	1	54.2	2
2.	EB-22	4	3	2	2	2	1	58.3	3
3.	EB-31	4	4	3	3	4	1	79.2	4
4.	EB-35	4	4	4	4	4	1	87.5	4
5.	EB-40	4	3	4	4	4	1	83.3	4
6.	EB-41	3	2	2	3	1	1	50.0	2
7.	EB-65	4	2	4	3	2	1	66.7	4
8.	EF-81	4	3	2	4	1	1	62.5	4
9.	Pf_1	4	2	2	4	3	1	66.7	4
10.	Pf_2	3	2	2	2	4	1	58.3	3
11.	Control	3	-	1	1	1	1	29.2	1

EB- Endophytic bacterium, EF- Endophytic fungus, Pf_1 – *P. fluorescens* (KAU), Pf_2 *P. fluorescens* (TNAU)
Score>25<45 = 1; >45<55= 2; >55< 60= 3 and >60 = 4

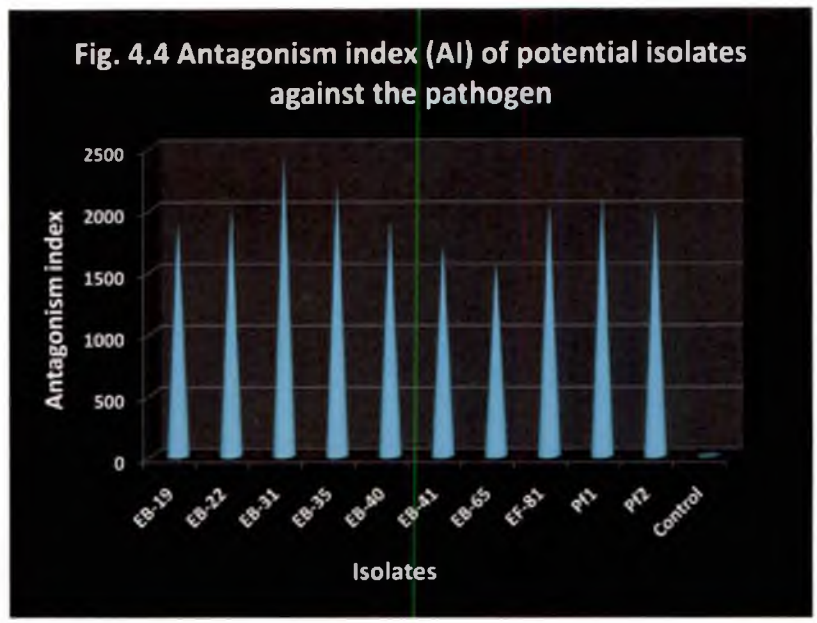
Fig. 4.4 Antagonism index (AI) of potential isolates against the pathogen
Antagonism index
2500
2000
1500
1000
500
0
EB-19
EB-22
EB-31
EB-35
EB-40
EB-41
EB-65
EF-81
PI1
PI2
Control
Isolates

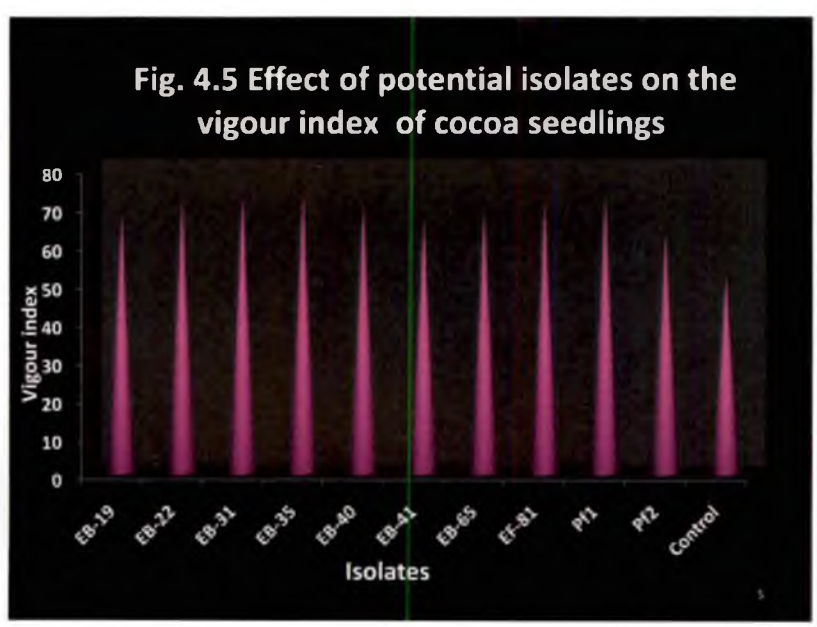
Fig. 4.5 Effect of potential isolates on the vigour index of cocoa seedlings
Vigour index
80
70
60
50
40
30
20
10
0
EB-19
EB-22
EB-31
EB-35
EB-40
EB-41
EB-65
EF-81
PI1
PI2
Control
Isolates

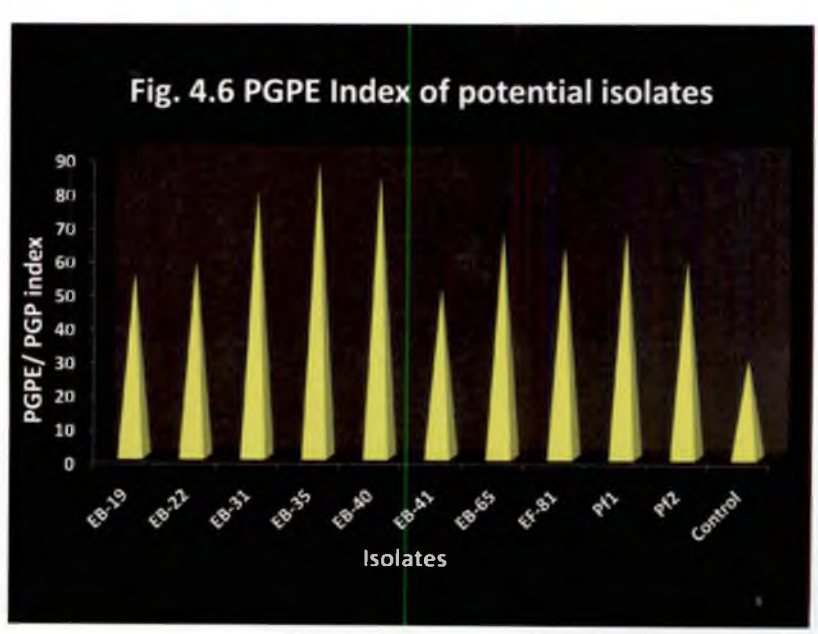
Fig. 4.6 PGPE Index of potential isolates
PGPE/ PGP index
90
80
70
60
50
40
30
20
10
0
EB-19
EB-22
EB-31
EB-35
EB-40
EB-41
EB-65
EF-81
PI1
PI2
Control
Isolates

4.6.8 Selection of promising endophytes

The eight potential antagonists were again short listed to five promising endophytes which recorded the maximum score for PGPE index. They are EB-31, EB-35, EB-40, EB-65 and EF-81 (Plate 4.8). These were subjected to further detailed investigation on their mechanisms of action and induction of systemic resistance.

4.6.9 Inhibition of the pathogen by production of volatile inhibitory metabolites

None of the endophytes tested was positive for HCN production. Hence, this test was done in order to know whether they produce any volatile compounds other than HCN. Data presented in Table 4.22 clearly showed that, upto 59.2 per cent inhibition of the pathogen was effected by EB-35 by way of volatile production. The per cent inhibition due to volatile metabolites varied from 18 (EF-81) to 63.1 (EB-35) on the third day after inoculation. At five days, the maximum inhibition (49.9 per cent) was recorded by EB-35 followed by Pf_2 (48.0 per cent) and EB-31 (34.3 per cent) whereas, after seven days of inoculation, the maximum inhibition of 59.2 per cent was recorded by EB-35 followed by EB-31 (54.4 per cent) (Plate 4.9). EB-40 and Pf_1 recorded a percentage inhibition of 44.8 and 46.7 per cent respectively. The least inhibition was with EF-81 (43.2).

4.6.10 Inhibition of the pathogen by diffusible, non-volatile metabolites

Production of non-volatilemetabolites by the promising endophytes in comparison with reference cultures was tested by the cellophane method as described in 3.6.9. The results of this experiment are furnished in Table 4.23. At three days after inoculation, the per cent inhibition of the pathogen varied from 20 to 57.4. The maximum inhibition was recorded by EB-35 followed by EB-31 (Plate 4.10) and Pf_2. The minimum was by EF-81 (20.0 per cent). The per cent inhibition varied from 22.6 to (EF-81) 39.6 (EB-40) at five days after inoculation and after seven days, the maximum inhibition was noticed in EB-35 and EB-40 (59.4) followed by EB-65 (58.3), EB-31 (54.4) and Pf_2 (51.7).

Table 4.22 *In vitro* inhibition of the pathogen by volatile inhibitory metabolites

Sl. No.	Isolate	3 DAI		5 DAI		7 DAI	
		Inhibition* (mm)	Per cent inhibition over control	Inhibition* (mm)	Per cent inhibition over control	Inhibition* (mm)	Per cent inhibition over control
1	EB-31	1.11	36.9	2.06	34.3	4.90	54.4
2	EB-35	1.89	63.1	2.99	49.9	5.33	59.2
3	EB-40	0.95	31.8	1.69	28.1	4.03	44.8
4	EB-65	1.20	39.9	1.87	31.1	4.83	53.7
5	EF-81	0.54	18.0	1.35	22.5	3.89	43.2
6	Pf_1	1.08	36.0	1.87	31.1	4.20	46.7
7	Pf_2	1.49	49.8	2.88	48.0	4.70	52.2

* Mean of three replications, EB-Endophytic bacterium, EF-Endophytic fungus, Pf_1 - *P. fluorescens* (KAU), Pf_2- *P. fluorescens* (TNAU), DAI-Days after inoculation

Table 4.23 *In vitro* inhibition of the pathogen by diffusible non-volatile inhibitory metabolites

Sl. No.	Isolate	3 DAI		5 DAI		7 DAI	
		Inhibition* (mm)	Per cent inhibition over control	Inhibition* (mm)	Per cent inhibition over control	Inhibition* (mm))	Per cent inhibition over control
1.	EB-31	1.10	36.8	1.93	32.1	4.90	54.4
2.	EB-35	1.72	57.4	2.15	35.8	5.35	59.4
3.	EB-40	1.01	33.8	2.38	39.6	5.35	59.4
4.	EB-65	0.97	32.4	2.21	36.8	5.25	58.3
5.	EF-81	0.60	20.0	1.36	22.6	4.47	49.7
6.	Pf_1	1.01	33.8	1.58	26.4	4.35	48.3
7.	Pf_2	1.10	36.8	1.93	32.1	4.65	51.7

* Mean of three replications, EB-Endophytic bacterium, EF-Endophytic fungus, Pf_1 - *P. fluorescens* (KAU), Pf_2- *P. fluorescens* (TNAU), DAI-Days after inoculation

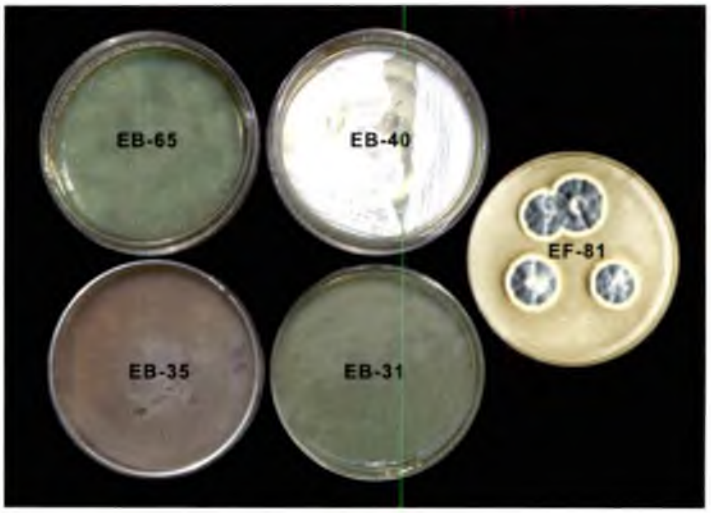

Plate 4.8 Promising endophytic isolates

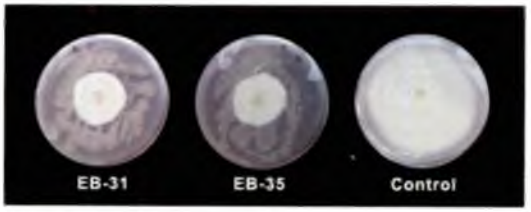

Plate 4.9 *In vitro* inhibition of the pathogen by volatile metabolites of endophytes

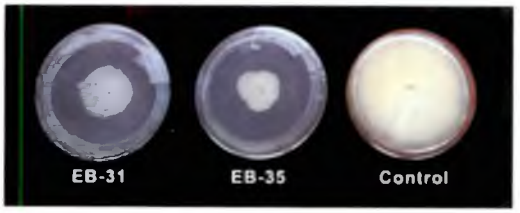

Plate 4.10 *In vitro* inhibition of the pathogen by non-volatile metabolites of endophytes

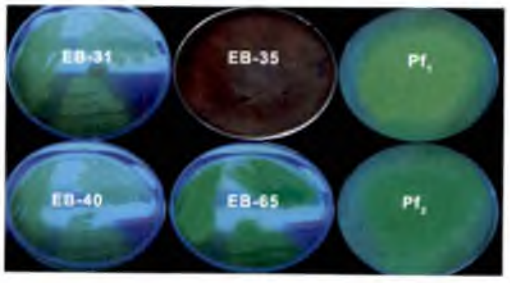

Plate 4.11 Fluorescence emitted by promising bacterial endophytes under UV light

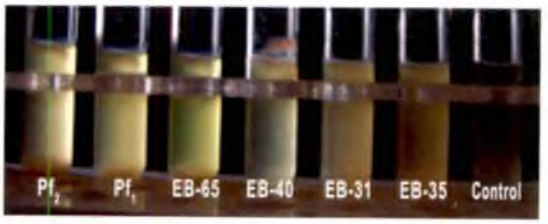

Plate 4.12 Siderophore production by promising bacterial endophytes

Pf_1- *P.fluorescens* (KAU)
Pf_2- *P.fluorescens* (TNAU)

4.6.11 Detection of fluorescence

The fluorescence emitted by the promising bacterial isolates were viewed under UV trans illuminator. It was observed that three among them *viz.*, EB-31 and EB-40 emitted yellow fluorescence while EB-65 emitted greenish fluorescence (Plate 4.11).

4.6.12 Iron dependant production of siderophores

The promising bacterial isolates were evaluated for iron dependent production of siderphores as per 3.6.12. Results of this experiment (Table 4.24) indicate that the endophytic isolates produce more siderophores when there is least amount of available iron in the medium. The siderophore released into the medium by the isolates decreased as the concentration of $FeCl_3$ increased. The isolate EB-65 produced the highest amount of siderophores with no $FeCl_3$ added followed by EB-31 (Plate 4.12). EB-35 showed the least amount of siderophores. When 100µM of $FeCl_3$ was present the production of siderophores by the isolates decreased however, maximum was with EB-65 followed by EB-31 and Pf_1 and the minimum was with EB-35. When 200 µM of $FeCl_3$ was present, the release of siderophores by the isolates further decreased and the maximum quantity of siderophores was released by EB-31 followed by EB-65, Pf_1 and EB-40. When the growth medium contained 400 µM of $FeCl_3$, the release of siderophores by EB-65 decreased to the half of that at the initial rate. Siderophores production by other isolates also decreased but the maximum was by EB-31, followed by EB-40 and Pf_1.

4.7 INDUCTION OF SYSTEMIC RESISTANCE IN COCOA

A pot culture experiment was laid out to evaluate effect of selected promising endophytes in inducing systemic resistance in cocoa in comparison with standard cultures of *Pseudomonas fluorescens* and chemicals used for control of seedling blight and *Phytophthora* pod rot of cocoa (Plate 4.13). Effect of the treatments on growth parameters and incidence and severity of seedling blight were also studied. Defence related compounds such as phenol, protein and enzymes were also estimated after

Plate 4.13 Views of the pot culture experiment for studying the induction of systemic resistance in cocoa seedlings

Plate 4.14 Effect of endophytes in reducing the infection on inoculation with the pathogen

challenge inoculation with the pathogen (Plate 4.14). The isozyme profile of defence related enzymes was assessed by native PAGE analysis.

4.7.1 Growth parameters

Growth parameters such as germination percentage, and biometric observations were recorded as per 3.7.3 and the results are presented below.

4.7.1.1 Germination percentage

The data presented in Table 4.25 revealed that, the treatments including promising endophytic isolates had a positive effect on germination of cocoa beans. The treatments had not only lead to increase in percentage germination but they had an effect on early germination also. Accordingly, cent per cent of the beans treated with EB-31 germinated on the eighth day after sowing compared to less than 50 per cent in the control. Treatments T_2 (EB-35) and T_7 (Pf_2) also recorded cent per cent germination by 12^{th} day after sowing. On the 14^{th} day, treatment T_3 (EB-40) also registered cent per cent germination whereas, 98 per cent of the beans germinated in T_4 (EB-65), T_8 (PP), 96 per cent in T_5 (EF-81) and T_6 (Pf_1)and 92 per cent in T_9 (BM) and only 86 per cent of the beans germinated in the control (T_{10}).

4.7.1.2 Height of cocoa seedlings

The treatments differed significantly in their effect on seedling height at one month after sowing (Table 4.26) with seedlings in T_8 (PP) exhibiting the maximum height followed by T_3 (EB-40), T_4 (EB-65), T_5 (EF-81), T_6 (Pf_1) and T_9(BM). However, these were on par with one another and also with control. The least height was for seedlings in treatment T_1 (EB-31). At the end of two months of sowing, there was no significant difference among the treatments whereas during three, and four months of sowing, there was significant difference in height among the treatments and T_1 (EB-31) and T_2 (EB-35) respectively showed the maximum height. At five months after sowing also, the treatment T_1 (EB-31) recorded the maximum seedling height and the minimum

Table 4.25 Effect of different treatments on germination of cocoa beans

Sl. No.	Treatment	8 DAS		10 DAS		12 DAS		14 DAS	
		No. of beans germinated	Per cent germination	No. of beans germinated	Per cent germination	No. of beans germinated	Per cent germination	No. of beans germinated	Per cent germination
1.	T_1 (EB-31)	50	100	50	100	50	100	50	100
2.	T_2 (EB-35)	41	82	45	90	50	100	50	100
3.	T_3 (EB-40)	45	90	47	94	48	96	50	100
4.	T_4 (EB-65)	38	76	41	82	42	84	49	98
5.	T_5 (EF-81)	21	42	23	56	33	66	48	96
6.	T_6 (Pf_1)	33	66	33	66	34	68	48	96
7.	T_7 (Pf_2)	42	84	49	98	50	100	50	100
8.	T_8 (PP)	41	82	43	86	47	94	49	98
9.	T_9 (BM)	27	54	41	82	41	82	46	92
10.	T_{10} (Control)	21	42	33	66	35	70	43	86

DAS-Days after sowing, EB-Endophytic bacterium, EF- Endophytic fungus, Pf_1-*P. fluorescens* (KAU), Pf_2 –*P. fluorescens* (TNAU), PP- Potassium phosphonate, BM - Bordeaux mixture

was in control and T_9(BM) (Fig. 4.7). At this stage, the maximum efficiency (43.5 per cent) in augmenting seedling height was exhibited by T_1 (EB-31) followed by T_6 (Pf_1) (32.8), T_2 (EB-35) and T_4 (EB-65) (32.6 per cent). The least effect on seedling height was showed by T_5(EF-81) and T_9(BM).

4.7.1.3 Number of leaves

The treatments exhibited a significant difference on the number of leaves at each stage of observation (Table 4.27). The number of leaves was the maximum for seedlings treated with T_1 (EB-31) throughout the period of observation except at one month after sowing. After one month of sowing, T_2 (EB-35) and T_4 (EB-65) recorded the maximum number of leaves but these were on par with all the other treatments except control. Treatments T_1 (EB-35), and T_9 (BM) followed by T_8 (PP), T_7 (Pf_2), T_6 (Pf_1) and T_2 (EB-35) recorded more number of leaves at the end two months of sowing and these were on par. At three, four and five months after sowing, the maximum number of leaves was present in T_1 (EB-31) (Fig. 4.7) and the minimum in plants treated with T_9 (BM). Seedlings treated with EB-31 had 50.6 per cent more leaves than the control at five months after sowing.

4.7.1.4 Girth at collar, fresh and dry weight of shoot

Observations on girth at collar region and fresh and dry weight of shoot of cocoa seedlings were recorded at five months after sowing (Table 4.28). The treatments had significant effect on all the three parameters studied. The treatment T_1 (EB-31) recorded the maximum girth with 73.3 per cent increase over control which was followed by T_7 (Pf_2), T_4, (EB-65) and T_5 (EF-81) (Fig. 4.8). The minimum girth was for seedlings treated with T_9 (BM), which had a negative effect on girth at collar. Maximum fresh weight of shoot was also recorded by T_1 (EB-31) (Fig. 4.8) which had 50.8 per cent more weight over control. It was followed by T_7 (Pf_2) which had 35.9 per cent efficiency in augmenting fresh weight and T_4 (EB-65) recorded 35.4 per cent more fresh weight. The minimum fresh weight of shoots was in T_9 (Bordeaux mixture). However, it was on par with all other treatments except T_1. Dry weight of shoot was maximum for

Table 4.26 Effect of different treatments on height of cocoa seedlings

Sl. No.	Treatment	Height of cocoa seedlings* (cm)					
		1MAS	2MAS	3MAS	4MAS	5MAS	Per cent +/- over control 5MAS
1.	T_1 (EB-31)	10.3^{b}	17.9^{a}	29.3^{a}	33.4^{b}	49.8^{a}	+ 43.5
2.	T_2 (EB-35)	12.3^{ab}	16.6^{a}	28.6^{a}	39.2^{a}	46.0^{ab}	+32.6
3.	T_3 (EB-40)	15.0^{a}	16.2^{a}	20.3^{c}	29.8^{bc}	39.0^{bc}	+12.1
4.	T_4 (EB-65)	15.0^{a}	16.4^{a}	18.9^{cd}	29.8^{bc}	46.0^{ab}	+32.6
5.	T_5 (EF-81)	14.3^{a}	17.1^{a}	18.3^{cd}	25.6^{cde}	36.2^{c}	+4.3
6.	T_6 (Pf_1)	12.6^{ab}	17.3^{a}	25.0^{b}	32.4^{b}	46.2^{ab}	+32.8
7.	T_7 (Pf_2)	13.7^{ab}	16.6^{a}	20.3^{c}	25.6^{cde}	40.8^{bc}	+17.6
8.	T_8 (PP)	15.2^{a}	15.8^{a}	16.6^{d}	38.6^{a}	38.8^{c}	+11.5
9.	T_9 (BM)	13.7^{ab}	15.6^{a}	17.6^{cd}	24.0^{de}	34.8^{c}	0
10.	T_{10}(Control)	12.6^{ab}	15.6^{a}	16.5^{d}	18.4^{e}	34.8^{c}	--

* Values followed by same super script are not significantly different, Mean of three replications, MAS-Months After sowing, EB-Endophytic bacterium, EF- Endophytic fungus, Pf_1-*P. fluorescens* (KAU), Pf_2 - *P. fluorescens* (TNAU), PP- Potassium phosphonate, BM.- Bordeaux mixture

Table 4.27 Effect of different treatments on number of leaves of cocoa seedlings

Sl. No.	Treatment	Number of leaves of cocoa seedlings* (cm)					
		1MAS	2MAS	3MAS	4MAS	5MAS	Per cent +/- over control 5MAS
1.	T_1 (EB-31)	3.0^{a}	6.0^{a}	15.2^{a}	16.4^{a}	23.2^{a}	+50.6
2.	T_2 (EB-35)	3.8^{a}	5.2^{a}	10.2^{b}	14.8^{ab}	17.2^{bc}	+11.7
3.	T_3 (EB-40)	3.6^{a}	3.8^{b}	11.6^{b}	14.7^{ab}	16.8^{bc}	+9.1
4.	T_4 (EB-65)	3.8^{a}	3.8^{b}	10.4^{b}	12.0^{bc}	$20:0^{b}$	+29.9
5.	T_5 (EF-81)	3.4^{a}	3.4^{b}	11.2^{b}	13.6^{abc}	16.0^{bc}	+3.9
6.	T_6 (Pf_1)	3.4^{a}	5.2^{a}	12.0^{b}	14.8^{ab}	18.0^{bc}	+16.9
7.	T_7 (Pf_2)	2.8^{ab}	5.6^{a}	12.0^{b}	13.2^{abc}	16.4^{bc}	+6.5
8.	T_8 (PP)	2.8^{ab}	5.8^{a}	10.8^{b}	12.4^{bc}	15.6^{bc}	+1.3
9.	T_9 (BM)	2.8^{ab}	6.0^{a}	10.4^{b}	11.6^{c}	14.8^{c}	-3.9
10.	T_{10}(Control)	1.8^{b}	3.4^{b}	9.6^{b}	11.6^{c}	15.4^{bc}	--

* Values followed by same super script are not significantly different, Mean of three replications, MAS-Months after sowing, EB-Endophytic bacterium, EF- Endophytic fungus, Pf_1-*P. fluorescens* (KAU), Pf_2 - *P. fluorescens* (TNAU), PP- Potassium phosphonate, BM - Bordeaux mixture

Table 4.28 Effect of different treatments on girth at collar, fresh and dry weight of shoot of cocoa seedlings

Sl. No.	Treatment	Girth at collar* (cm) (5MAS)	Per cent +/- over control	Fresh weight* (g) (5MAS)	Per cent +/- over control	Dry weight* (g) (5MAS)	Per cent +/- over control
1.	T_1 (EB-31)	5.2 ^{a}	+73.3	39.5 ^{a}	+50.8	10.1 ^{a}	+106.1
2.	T_2 (EB-35)	3.2 ^{b}	+6.7	33.6 ^{bc}	+28.2	5.6 ^{bc}	+14.3
3.	T_3 (EB-40)	3.6 ^{ab}	+20.0	33.9 ^{bc}	+29.3	6.4 ^{abc}	+30.6
4.	T_4 (EB-65)	4.2 ^{a}	+40.0	35.4 ^{bc}	+35.1	8.9 ^{ab}	+81.6
5.	T_5 (EF-81)	4.1 ^{a}	+36.7	34.9 ^{bc}	+33.2	8.4 ^{abc}	+71.4
6.	T_6 (Pf_1)	3.7 ^{ab}	+23.3	34.8 ^{bc}	+32.8	7.6 ^{abc}	+55.1
7.	T_7 (Pf_2)	4.3 ^{a}	+43.3	35.6 ^{bc}	+35.9	10.0 ^{a}	+104.1
8.	T_8 (PP)	3.7 ^{ab}	+23.3	34.9 ^{bc}	+33.2	8.2 ^{abc}	+67.3
9.	T_9 (BM)	2.9 ^{b}	-3.3	20.4 ^{c}	-22.1	4.6 ^{c}	-6.1
10.	T_{10}(Control)	3.0 ^{b}	--	26.2 ^{bc}	--	4.9 ^{c}	--

* Values followed by same super script are not significantly different, Mean of three replications, MAS-Months after sowing, EB-Endophytic bacterium, EF- Endophytic fungus, Pf_1-*P. fluorescens* (KAU), Pf_2 - *P. fluorescens* (TNAU), PP- Potassium phosphonate, BM - Bordeaux mixture

T_1 (EB-31) followed by T_7 (Pf_2) and these were on par with five other treatments (Fig. 4.8). T_1 (EB-31) recorded 106.1 per cent efficiency in increasing dry weight followed by T_7 (Pf_2) with 104.1 per cent efficiency. The minimum dry weight was for seedlings treated with T_9 (BM).

4.7.1.4 Root length and fresh and dry weight of root

Observations on root length and fresh and dry weight of roots were recorded at five months after sowing (Table 4.29). The treatments had significant effect on the three parameters studied (Fig. 4.9). T_2 (EB-35) recorded the maximum root length which was followed by T_5 (EF-81), T_3 (EB-40) and T_7 (Pf_2) and these four treatments were on par. T_2 (EB-35) had 56.5 per cent increase in root length and T_5 (EF-81), T_3(EB-40) and T_7(Pf_2) had 43.5, 41.1 and 35.3 per cent more root length respectively compared to control. The least root length was recorded in control. All the treatments except T_9 (BM) recorded significantly higher fresh weight of roots over control. T_1 (EB-31) and T_4 (EB-65) recorded the maximum fresh weight with 269 per cent increase over control, closely followed by T_7 (Pf_2) with 267 per cent increase. However, these were on par with six other treatments. T8 (PP) also had significantly higher fresh weight with 58 per cent increase over control. The minimum fresh weight of roots was in T_9 (Bordeaux mixture). Dry weight of roots was maximum for T_4 (EB-65) which had 111.8 per cent increase followed by T_1 (EB-31) with 100 per cent increase and these were on par with T_7 (Pf_2), T_8 (PP), T_6 (Pf_1), T_2 (EB-35) and T_3 (EB-40) also.

4.7.2 Effect of different treatments on seedling blight of cocoa

The incidence and severity of seedling blight upon challenge inoculation was recorded at periodic intervals (Table 4.30). The disease incidence varied from 27 to 30.0 per cent at three days after inoculation and there was no significant difference among the treatments. However, at five days after inoculation, there was cent per cent incidence of the disease in control and the various treatments showed a significant difference. The least incidence of the disease (43.3) was in T_1 (EB-31) followed by T_2 (EB-35) (46.6).

Table 4.29 Effect of different treatments on length, fresh and dry weight of roots of cocoa seedlings

Sl. No.	Treatment	Root length* (cm) (5MAS)	Per cent +/- over control	Fresh weight* (g) (5MAS)	Per cent +/- over control	Dry weight* (g) (5MAS)	Per cent +/- over control
1.	T_1 (EB-31)	19.0 ^{de}	+11.8	14.4 ^{a}	+269.0	3.4 ^{a}	+100.0
2.	T_2 (EB-35)	26.6^{a}	+56.5	11.3 ^{a}	+190.1	2.3 ^{abcd}	+35.3
3.	T_3 (EB-40)	24.0^{abc}	+41.1	12.3 ^{a}	+215.3	2.3 ^{abcd}	+35.3
4.	T_4 (EB-65)	20.0^{cde}	+17.6	14. 4 ^{a}	+269.0	3.6^{a}	+111.8
5.	T_5 (EF-81)	24.4^{abc}	+43.5	10.1^{a}	+158.9	2.1 ^{bcde}	+23.5
6.	T_6 (Pf_1)	20.2 ^{bcde}	+18.8	12.1 ^{a}	+210.2	2.8 ^{abc}	+64.7
7.	T_7 (Pf_2)	23.0^{abcd}	+35.3	14.3 ^{a}	+267.0	3.2 ^{ab}	+88.2
8.	T_8 (PP)	18.8^{de}	+9.4	9.7 ^{a}	+58.0	3.1 ^{ab}	+82.4
9.	T_9 (BM)	17.6^{de}	+3.5	3.7 ^{b}	-5.1	1.9 ^{bcde}	+11.8
10.	T_{10}(Control)	17.0^{e}	--	3.9 ^{b}	--	1.7 ^{e}	--

* Values with same super script are not significantly different, Mean of three replications, MAS-Months after sowing, EB-Endophytic bacterium, EF-Endophytic fungus, Pf_1-*P. fluorescens* (KAU), Pf_2 - *P. fluorescens* (TNAU), PP- Potassium phosphonate, BM - Bordeaux mixture

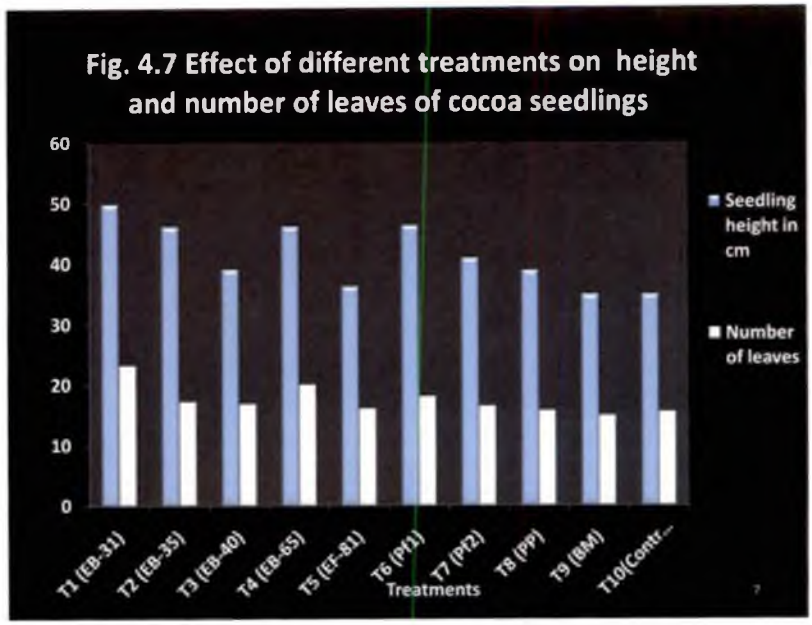

Fig. 4.7 Effect of different treatments on height and number of leaves of cocoa seedlings

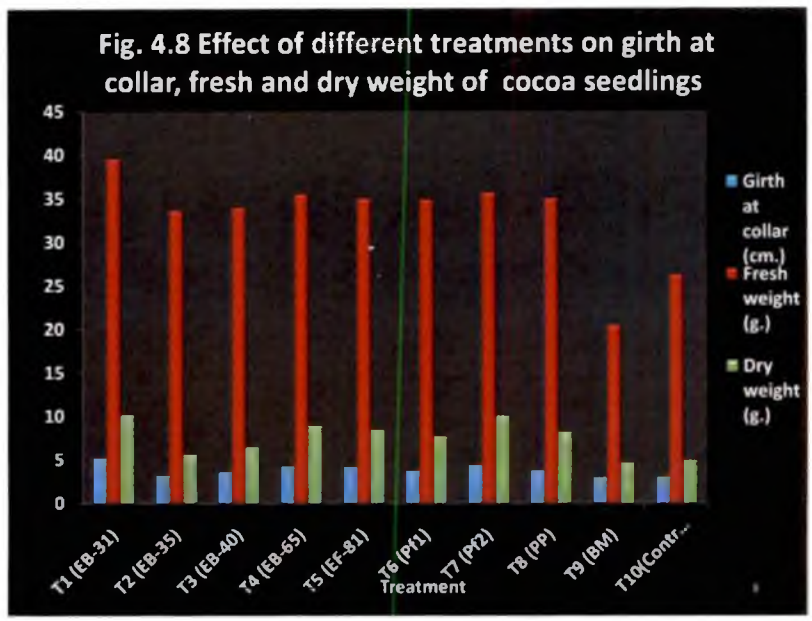

Fig. 4.8 Effect of different treatments on girth at collar, fresh and dry weight of cocoa seedlings

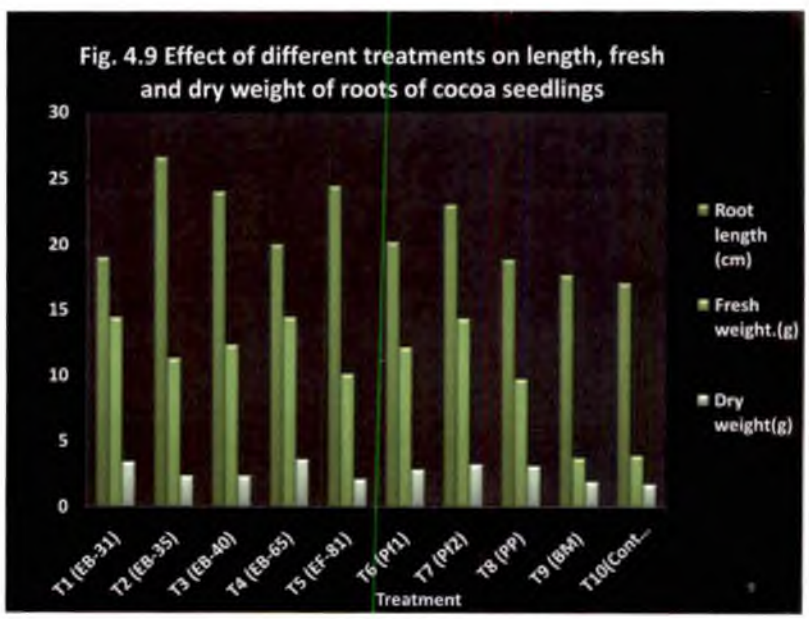

Fig. 4.9 Effect of different treatments on length, fresh and dry weight of roots of cocoa seedlings

Table 4.30 Effect of different treatments on incidence and severity of seedling blight of cocoa

Sl. No.	Treatment	3DAI		5DAI	
		Per cent Disease Incidence*	Per cent Disease Severity*	Per cent Disease Incidence	Per cent Disease Severity
1.	T_1 (EB-31)	27[a]	4.3[a]	43.3 (6.5)[a]	5.1 (1.9)[a]
2.	T_2 (EB-35)	30[a]	5.3[a]	46.6 (6.9)[ab]	11.9 (3.4)[bc]
3.	T_3 (EB-40)	27[a]	4.7[a]	70.0 (8.4)[b]	8.2 (2.9)[ab]
4.	T_4 (EB-65)	30[a]	4.9[a]	83.3 (9.1)[b]	12.9 (3.6)[bc]
5.	T_5 (EF-81)	30[a]	5.0[a]	90.0 (9.5)[b]	19.4 (4.4)[cd]
6.	T_6 (Pf_1)	29[a]	4.7[a]	50.0 (7.1)[ab]	12.4 (3.6)[bc]
7.	T_7 (Pf_2)	27[a]	5.3[a]	80.0 (8.9)[b]	11.3 (3.4)[bc]
8.	T_8 (PP)	33[a]	5.3[a]	96.7 (9.8)[b]	20.1 (4.5)[cd]
9.	T_9 (BM)	27[a]	4.9[a]	93.3 (9.6)[b]	24.4 (4.9)[d]
10.	T_{10} (Control)	30[a]	5.3[a]	100.0 (10.0)[b]	28.9 (5.4)[d]

* Values followed by same super script are not significantly different, Mean of three replications, DAI-Days after inoculation, EB-Endophytic bacterium, EF- Endophytic fungus, Pf_1-*P. fluorescens* (KAU), Pf_2 - *P. fluorescens* (TNAU), PP- Potassium phosphonate, BM - Bordeaux mixture

Table 4.31 Changes in total phenol content of cocoa leaves on challenge inoculation

Sl. No.	Treatment	Before inoculation		1 DAI		3 DAI		5 DAI	
		Total phenol µg g^{-1}	Per cent +/- over control	Total phenol µg g^{-1}	Per cent +/- over control	Total phenol µg g^{-1}	Per cent +/- over control	Total phenol µg g^{-1}	Per cent +/- over control
1.	T_1 (EB-31)	362.3^{bc}	12.27	609.0^{bcd}	43.86	835.0^{bc}	35.26	1027.3^{bc}	42.42
2.	T_2 (EB-35)	635.3^{a}	96.87	872.7^{ab}	106.16	1461.7^{a}	136.78	1566.0^{a}	117.10
3.	T_3 (EB-40)	425.0^{bc}	31.70	752.3^{abc}	77.72	944.3^{bc}	52.97	1229.0^{abc}	70.38
4.	T_4 (EB-65)	706.7^{a}	118.96	931.7^{a}	120.10	1192.7^{ab}	91.96	1282.3^{ab}	77.77
5.	T_5 (EF-81)	343.0^{d}	6.29	533.0^{cd}	25.91	741.7^{c}	20.15	783.3^{bc}	8.59
6.	T_6 (Pf_1)	383.7^{bc}	18.68	522.7^{cd}	23.48	820.3^{bc}	32.88	1012.0^{bc}	40.30
7.	T_7 (Pf_2)	344.7^{d}	6.78	533.7^{cd}	26.08	773.7^{bc}	25.33	985.3^{bc}	36.60
8.	T_8 (PP)	535.3^{ab}	65.78	713.7^{abcd}	68.60	944.0^{bc}	52.92	1242.7^{abc}	72.28
9.	T_9 (BM)	296.0^{d}	-8.27	460.7^{cd}	8.83	654.7^{c}	6.05	923.3^{bc}	28.00
10.	T_{10} (Control)	322.7^{d}	--	423.3^{d}	--	617.3^{c}	--	721.3^{c}	--

* Values followed by same super script are not significantly different, Mean of three replications, DAI-Days after inoculation, EB-Endophytic bacterium, EF- Endophytic fungus, Pf_1-*P. fluorescens* (KAU), Pf_2 - *P. fluorescens* (TNAU), PP- Potassium phosphonate, BM - Bordeaux mixture

Table 4.32 Changes in protein content of cocoa leaves on challenge inoculation

Sl. No.	Treatment	Before Inoculation		1 DAI		3 DAI		5 DAI	
		Total protein mg g^{-1}	Per cent +/- over control	Total protein mg g^{-1}	Per cent +/- over control	Total protein mg g^{-1}	Per cent +/- over control	Total protein mg g^{-1}	Per cent +/- over control
1.	T_1 (EB-31)	1.62 ^{cd}	15.71	2.65 ^{cd}	51.42	2.77 ^{b}	-15.03	2.74 ^{ab}	33.65
2.	T_2 (EB-35)	2.67 ^{ab}	90.71	5.37 ^{a}	206.85	4.59 ^{a}	40.79	3.67 ^{a}	79.02
3.	T_3 (EB-40)	2.27 ^{abcd}	62.14	4.81 ^{ab}	175.42	4.72 ^{a}	44.78	3.60 ^{a}	75.60
4.	T_4 (EB-65)	2.66 ^{ab}	90.00	3.70 ^{bc}	111.42	3.67 ^{ab}	12.57	3.65 ^{a}	78.04
5.	T_5 (EF-81)	1.64 ^{cd}	17.14	2.12 ^{d}	21.14	2.68 ^{b}	-17.79	2.90 ^{ab}	40.97
6.	T_6 (Pf_1)	2.40 ^{abc}	71.42	2.91 ^{cd}	66.85	3.67 ^{ab}	12.57	3.88 ^{a}	89.26
7.	T_7 (Pf_2)	2.78 ^{a}	98.57	3.28 ^{cd}	87.42	4.21 ^{ab}	29.14	3.13 ^{ab}	52.68
8.	T_8 (PP)	1.78 ^{bcd}	27.14	2.23 ^{cd}	27.42	3.50 ^{ab}	07.36	2.44 ^{ab}	19.02
9.	T_9 (BM)	1.54 ^{cd}	10.00	2.07 ^{d}	18.28	3.84 ^{ab}	17.79	3.04 ^{ab}	48.29
10.	T_{10} (Control)	1.39 ^{d}	--	1.75 ^{d}	--	3.26 ^{ab}	--	2.05 ^{b}	--

* Values followed by same super script are not significantly different, Mean of three replications, DAI-Days after inoculation, EB-Endophytic bacterium, EF- Endophytic fungus, Pf_1-*P. fluorescens* (KAU), Pf_2 - *P. fluorescens* (TNAU), PP- Potassium phosphonate, BM - Bordeaux mixture

and T_6 (Pf_1) (50.0) and these were on par. The rest of the treatments recorded disease incidence of 70 (T_3,EB-40) or above. Per cent disease incidence of 90 or above was recorded by treatments T_8 (PP), T_9 (BM), and T_5 (EF-81), while 80 to 90 per cent incidence was recorded by T_4 (EB-65), and T_7 (Pf_2). However, these treatments were on par with the control.

The per cent disease severity at three days after inoculation also did not show significant difference which varied from 4.3 to 5.3 (Table 4.30). The minimum severity was noticed in T_1 (EB-31) followed by T_3(EB-40) and T_6 (Pf_1) and the maximum in T_2 (EB-35), T_7 (Pf_2), T_8 (PP), and T_{10} (Control). After five days of inoculation, there was significant difference in disease severity. The minimum disease severity (5.1) was recorded by T_1 (EB-31) followed by T_3 (EB-40) (8.2) which were on par. The maximum severity (28.9 per cent) was recorded by T_{10} (control). However, the endophytic isolates and the two reference cultures (Pf_1 and Pf_2) recorded a severity percentage of less than twenty while the chemicals *viz.*, T_8 (PP) and T_9 (BM) had less than 25 per cent severity. Among the endophytes, the maximum severity was noticed in T_5 EF 81 (19.4 per cent).

4.7.3 Assay of defence related compounds and enzymes

Changes in the defence related compounds such as phenol, protein, and enzymes were studied before and after challenge inoculation with the pathogen. The results are furnished below.

4.7.3.1 Total phenol content

Content of total phenols in cocoa leaves was estimated at periodic intervals after challenge inoculation with the pathogen. The results presented in Table 4.31 showed that, phenol content in the leaves of cocoa seedlings varied from 296 to 706.7 $\mu g\ g^{-1}$ of fresh leaf tissue when assayed before inoculation with the pathogen. There was significant difference in phenol content among the treatments before challenge inoculation. T_4 (EB-65) recorded the highest phenol content, which had a per cent increase of 118.96 over control followed by T_2 (EB-35) with an increase of 96.87 per

cent. These two treatments were on par with each other and with T_8 (potassium phosphonate). Before inoculation, all the treatments except T_9 (Bordeaux mixture) had more phenol content than control. The total phenol content in leaves increased from the first day to fifth day after inoculation in all the treatments (Fig. 4.10). At one DAI, the phenol content was higher than that of control in all the treatments, when, the highest content was observed in plants treated with T_4 (EB-65) with 120.1 per cent more phenol than control. On the third day, the highest content was observed in T_2 (EB-35) followed by T_4 (EB-65) which were on par. At three DAI also, the lowest content was present in T_{10} (Control). However on fifth day, the highest content was recorded by T_2 (EB-35) followed by T_4 (EB-65), T_8 (PP) and T_3 (EB-40) respectively and these treatments were on par. The per cent increase of more than 100 per cent over control was seen only in T_2 (EB-35) (117.1 per cent) on fifth day. However all the treatments had more phenol content than control at five DAI also.

4.7.3.2 Protein content

Total protein content of cocoa leaves from different treatments was estimated before and at periodical intervals after challenge inoculation and the results are furnished in Table 4.32. In general the protein content in the leaves increased from before inoculation to 3 DAI (Fig. 4.11). Before inoculation, the protein content varied from 1.39 (control) to 2.78 mg g^{-1} of leaf tissue and there was a significant difference among the treatments. The highest content of protein was recorded in T_7 (Pf_2) with 98.57 per cent increase over control and it was on par with T_2 (EB-35), T_6 (Pf_1), T_4 (EB-65) and T_3 (EB-40). At one DAI, there was an increase in protein content in all the treatments compared to before inoculation. The highest content was with T_2 (EB-35), followed by T_3 (EB-40) which were on par. The increase over control was more than 100 per cent in three treatments they are T_2 (206.85), T_3 (175.42) and T_4 (111.42). The least content was recorded in T_{10} (control). From 1 DAI to 3 DAI there was decline in protein content in the leaves in three treatments *viz.*, T_3 (EB-40) and T_2 (EB-35) and T_4 (EB-65). However at 3 DAI, treatments T_3 (EB-40) and T_2 (EB-35) maintained the higher level of protein content compared to others with an increase of 44.78 and 40.79 per cent over control

Fig. 4.10 Changes in total phenol content of cocoa leaves on challenge inoculation

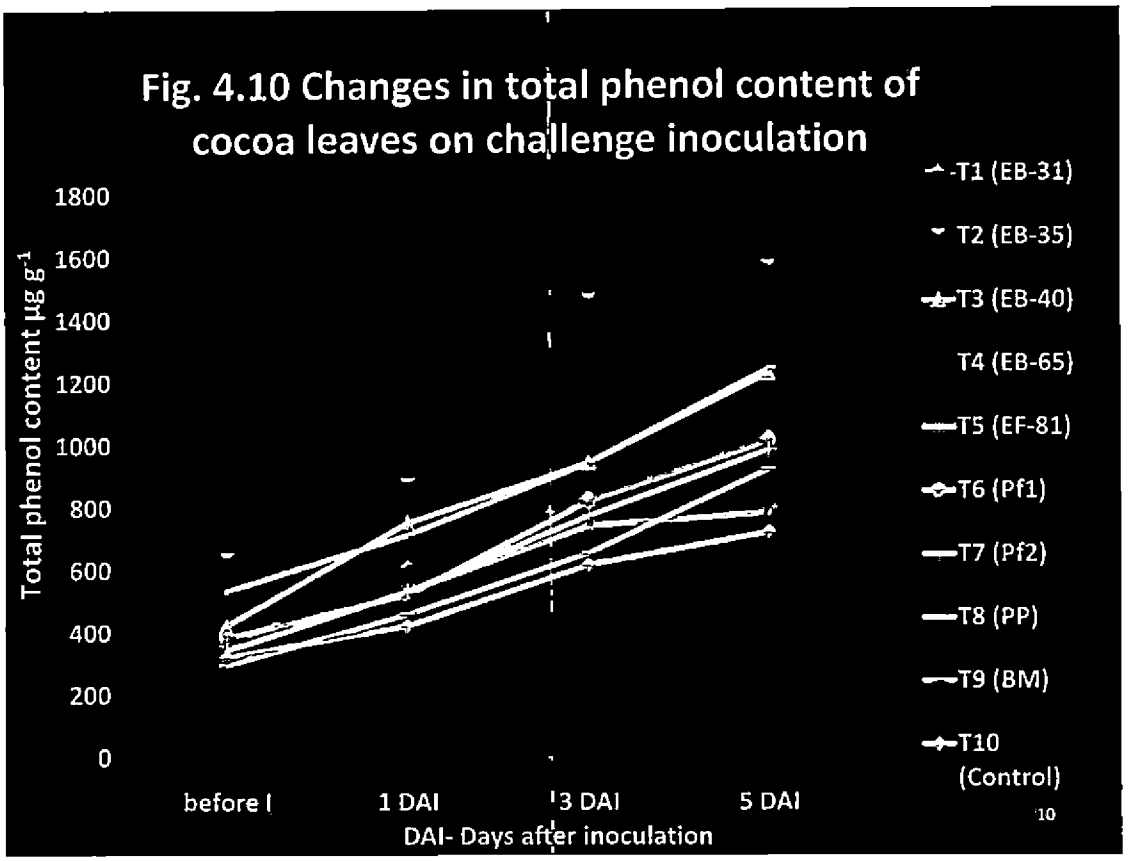

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Fig. 4.11 Changes in protein content of cocoa leaves on challenge inoculation

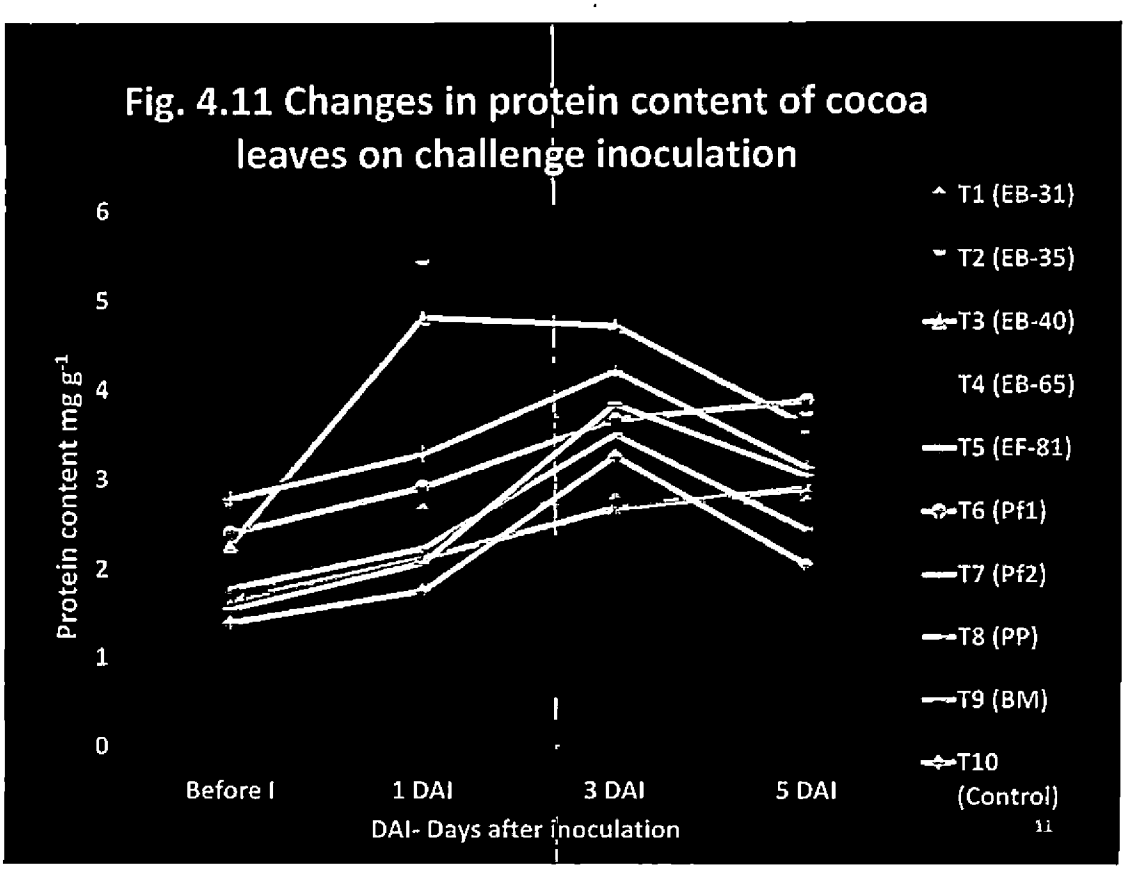

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Table 4.33 Effect of different treatments on the activity of peroxidase (PO) in cocoa leaves

Sl. No.	Treatment	Before		1 DAI		3 DAI		5 DAI	
		PO activity Δ_{436} $min^{-1}g^{-1}$ fresh tissue	Per cent +/- over control	PO activity Δ_{436} $min^{-1}g^{-1}$ fresh tissue	Per cent +/- over control	PO activity Δ_{436} $min^{-1}g^{-1}$ fresh tissue	Per cent +/- over control	PO activity Δ_{436} $min^{-1}g^{-1}$ fresh tissue	Per cent +/- over control
1.	T_1 (EB-31)	1.30	26.2	1.83	46.4	2.65	8.2	2.85	20.7
2.	T_2 (EB-35)	1.51	45.6	1.58	26.4	2.69	9.8	3.75	58.9
3.	T_3 (EB-40)	1.50	45.4	2.01	60.0	3.61	47.3	3.70	56.8
4.	T_4 (EB-65)	1.70	65.4	1.90	52.0	2.42	-1.2	2.50	5.9
5.	T_5 (EF-81)	1.07	3.9	1.30	4.0	2.63	7.3	2.65	12.3
6.	T_6 (Pf_1)	1.55	50.5	1.61	28.0	2.91	18.8	3.10	31.4
7.	T_7 (Pf_2)	1.30	26.2	1.88	50.4	2.85	16.3	3.90	65.3
8.	T_8 (PP)	1.05	1.9	1.76	40.8	3.06	24.9	3.30	39.8
9.	T_9 (BM)	1.04	0.9	1.25	0.00	2.05	-12.2	2.10	-11.0
10.	T_{10} (Control)	1.03		1.25		2.45		2.36	

DAI-Days after inoculation, EB-Endophytic bacterium, EF-Endophytic fungus, Pf_1-*P. fluorescens* (KAU), Pf_2-*P. fluorescens* (TNAU), PP-Potassium phosphonate, BM-Bordeaux mixture

Table 4.34 Effect of different treatments on the activity of polyphenol oxidase (PPO) in cocoa leaves

Sl. No.	Treatment	Before		1 DAI		3 DAI		5 DAI	
		PPO activity Δ_{420} $min^{-1}g^{-1}$ fresh tissue	Per cent +/- over control	PPO activity Δ_{420} $min^{-1}g^{-1}$ fresh tissue	Per cent +/- over control	PPO activity Δ_{420} $min^{-1}g^{-1}$ fresh tissue	Per cent +/- over control	PPO activity Δ_{420} $min^{-1}g^{-1}$ fresh tissue	Per cent +/- over control
1.	T_1 (EB-31)	0.67	116.1	2.33	86.4	2.65	13.7	1.51	48.0
2.	T_2 (EB-35)	1.51	387.1	2.71	116.8	2.75	18.0	1.23	20.6
3.	T_3 (EB-40)	1.71	451.6	2.05	64.0	2.90	24.5	1.11	8.8
4.	T_4 (EB-65)	1.62	422.6	2.03	62.4	2.25	-3.4	1.01	-9.8
5.	T_5 (EF-81)	1.15	270.9	1.40	12.0	1.85	-20.6	1.34	31.4
6.	T_6 (Pf_1)	1.30	319.4	2.85	128.0	3.10	33.0	1.30	27.5
7.	T_7 (Pf_2)	1.05	241.9	2.25	80.0	2.65	13.7	1.15	12.7
8.	T_8 (PP)	0.77	148.4	2.15	72.0	1.65	-29.2	1.12	9.8
9.	T_9 (BM)	0.31	0	1.24	0	2.34	0	1.02	0
10.	T_{10} (Control)	0.31	--	1.25	--	2.33	--	1.02	--

DAI-Days after inoculation, EB-Endophytic bacterium, EF-Endophytic fungus, Pf1-*P. fluorescens* (KAU), Pf_2-*P. fluorescens* (TNAU), PP-Potassium phosphonate, BM-Bordeaux mixture

respectively. The content of protein in leaves decreased from 3DAI to 5DAI in all the treatments. Nevertheless, the content was higher than control in all. The maximum increase over control was recorded by T_6 (Pf_1) (89.26) and the least by T_8 (PP) (19.02) at 5DAI.

4.7.4 Assay of defence related enzymes

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

4.7.4.1 Peroxidase (PO)

Application of the various treatments resulted in an increase in the activity of PO (Table 4.33). The activity of PO as expressed by the change in absorbance ranged from 1.03 (T_{10}) (control) to 1.7 (T_4) (EB-65) before inoculation. Treatment T_4 (EB-65) recorded 65.4 per cent more activity of PO over control before inoculation which was the highest. Next to this, T_6 (Pf_1) gave an increase in PO activity by 50.5 per cent over control which was followed by 45.6 and 45.4 per cent increase by treatments T_2 (EB-35) and T_3 (EB-40) respectively whereas, the treatment with the chemicals *viz.*, T_8 (PP) and T_9 (BM) recorded low increase in PO activity by 1.9 and 1.8 per cent respectively. In general, the activity increased in all the treatments from the first to the fifth day after inoculation (Fig. 4.12). At 1 DAI, activity of PO was the maximum in T_3 (EB-40) which had 60 per cent efficiency in augmenting PO activity. It was followed by T_4 (EB-65) with 52 per cent efficiency. The minimum activity of PO was in control and T_5 (EF-81). At 3 DAI, the activity of PO was the maximum and 47.3 per cent more than that of control in T_3 (EB 40) followed by T_8 (PP) with 24.9 per cent increase and T_6 (Pf_1) with 18.8 per cent increase. While the activity was less than that of control in T_4 (EB-65) at 3DAI. From 3 DAI to 5 DAI, the PO activity increased in all the treatments except control with 65.3 per cent more activity over control in T_7 (Pf_2) at five DAI. More than fifty per cent increase over control was recorded by T_2 (EB-35) and T_3 (EB-65) and

more than 30 per cent increase by T_8 (PP) and T_6 (Pf_1). However, the activity was less than control in T_9 (BM) at 5 DAI.

4.7.4.2 Polyphenol oxidase (PPO)

The activity of PPO in all the treatments showed an increasing trend from before inoculation to third day after inoculation and there after it declined (Table 4.34). Higher activity of PPO over control was noticed in all the treatments before inoculation with a maximum increase of 451.6 per cent in T_3 (EB-40) and minimum of 116.1 per cent in T_1 (EB-31). Out of the two chemicals tested, potassium phosphonate (T_8) showed higher activity of PPO while in plants treated with Bordeaux mixture the activity was more or less the same as that in control (Fig. 4.13). There was a sharp increase in PPO activity from before inoculation to 1 DAI when, it was the maximum in plants treated with Pf_1 followed by EB-35, EB-31 and Pf_2. On the third day after inoculation also, treatments T_6 (Pf_1) (33.0), T_3 (EB-40) (24.5), T_2 (EB-35) (18.0), T_1(EB-31)and T_7 (Pf_2) (13.7) showed more activity of PPO than that of control but the per cent increase over control was less compared to 1DAI. T_6 (Pf_1) recorded the highest activity of PPO on the third day and the least was in T_8 (PP). From 3DAI to 5DAI, activity of PPO showed declining trend however, four endophytic isolates *viz.*, EB-31, EB-35, EB-40 and EF-81 and the two reference cultures had effected more PPO than control at 5DAI. The maximum increase over control was with T_1 (EB-31) followed by T_5 (EF-81).

4.7.4.3 β-1, 3-glucanase

Activity of β-1, 3-glucanase was studied before, one DAI and three DAI (Table 4.35). It showed an increasing trend from before inoculation to 1 DAI and there after declined (Fig. 4.14). The activity before inoculation was the maximum in T_6 (Pf_1) followed by T_5 (EF-81), T_1 (EB-31), and T_4 (EB-40). All the treatments showed an increase in the activity compared to control and the per cent increase was 50.7 in T_6 (Pf_1). However, the treatments T_6 (Pf_1), T_1 (EB-31), T_5 (EF-81), T_3 (EB-40) and T_7 (Pf_2) showed more than 20 per cent more activity compared to control, while the increase was 15.3 per cent in T_2 (EB-35), 13.9 in T_8 (PP), 13.7 in T_4 (EB-65) and only 3.2 per cent in

Table 4.35 Effect of different treatments on the activity β -1, 3 - glucanase in cocoa leaves

Sl.No	Treatment	Before Inoculation		1 DAI		3 DAI	
		activity units mg^{-1} leaf tissue	Per cent +/- over control	activity units mg^{-1} leaf tissue	Per cent +/- over control	activity units mg^{-1} leaf tissue	Per cent +/- over control
1.	T_1 (EB-31)	89.71	38.9	139.8	66.4.	130.8	57.7
2.	T_2 (EB-35)	74.50	15.3	135.3	61.1	128.2	54.6
3.	T_3 (EB-40)	81.33	25.9	122.0	45.2	111.9	34.9
4.	T_4 (EB-65)	73.41	13.7	116.4	38.6	109.6	32.2
5.	T_5 (EF-81)	92.38	43.1	148.2	76.4	144.8	74.7
6.	T_6 (Pf_1)	97.31	50.7	161.2	77.2	152.4	83.8
7.	T_7 (Pf_2)	77.60	20.2	115.4	37.4	103.2	24.5
8.	T_8 (PP)	73.54	13.9	90.8	7.2	83.8	1.1
9.	T_9 (BM)	66.65	3.2	85.2	1.4	82.9	0
10.	T_{10} (Control)	64.57	--	84.0	--	82.9	--

DAI - Days after inoculation, EB-Endophytic bacterium, EF-Endophytic fungus, Pf_1-*P. fluorescens* (KAU), Pf_2-*P. fluorescens* (TNAU), PP-Potassium phosphonate, BM-Bordeaux mixture

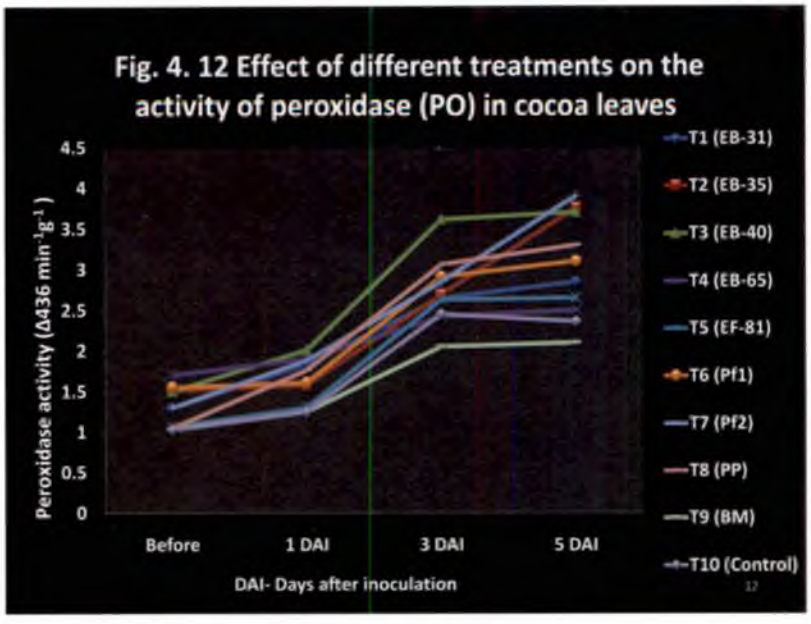
Fig. 4. 12 Effect of different treatments on the activity of peroxidase (PO) in cocoa leaves
Peroxidase activity (Δ436 min-1g-1)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Before
1 DAI
3 DAI
5 DAI
DAI- Days after inoculation
T1 (EB-31)
T2 (EB-35)
T3 (EB-40)
T4 (EB-65)
T5 (EF-81)
T6 (Pf1)
T7 (Pf2)
T8 (PP)
T9 (BM)
T10 (Control)
12

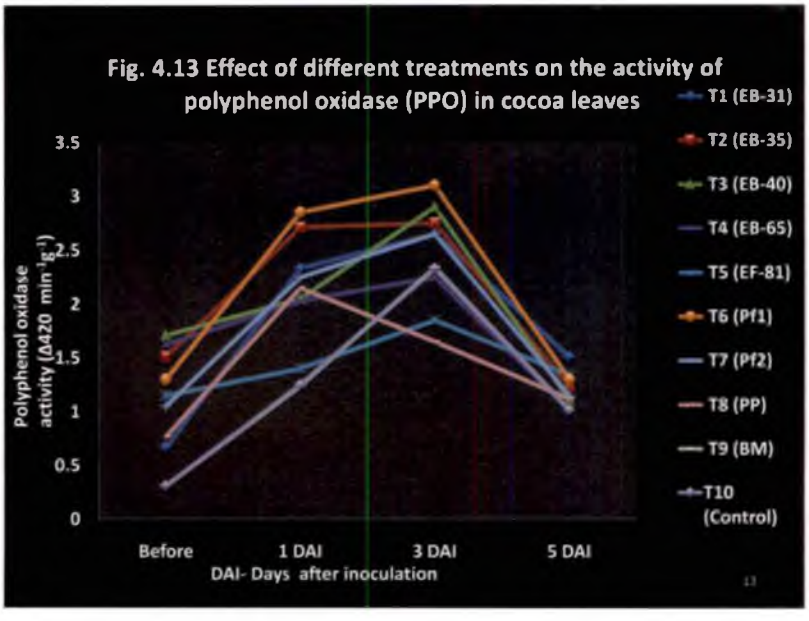
Fig. 4.13 Effect of different treatments on the activity of polyphenol oxidase (PPO) in cocoa leaves
Polyphenol oxidase activity (Δ420 min-1g-1)
3.5
3
2.5
2
1.5
1
0.5
0
Before
1 DAI
3 DAI
5 DAI
DAI- Days after inoculation
T1 (EB-31)
T2 (EB-35)
T3 (EB-40)
T4 (EB-65)
T5 (EF-81)
T6 (Pf1)
T7 (Pf2)
T8 (PP)
T9 (BM)
T10 (Control)
13

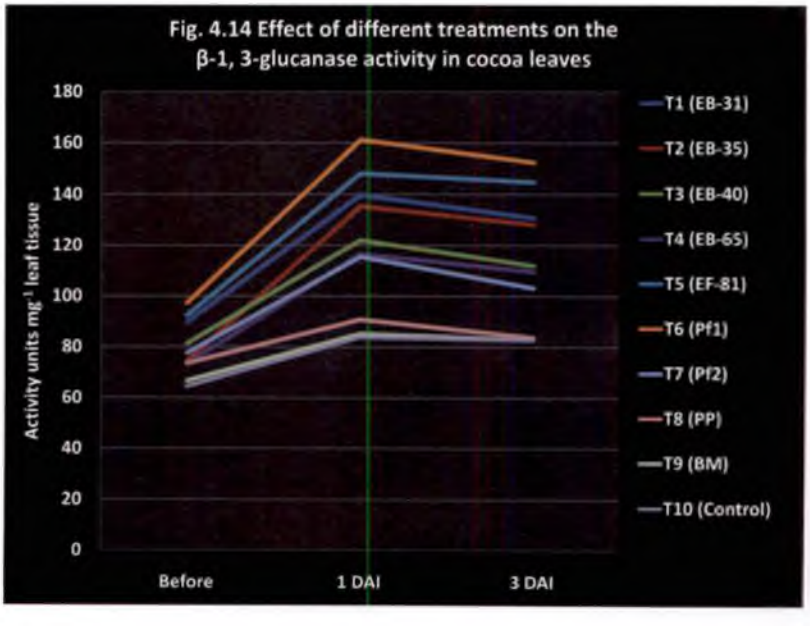
Fig. 4.14 Effect of different treatments on the β-1, 3-glucanase activity in cocoa leaves
Activity units mg-1 leaf tissue
180
160
140
120
100
80
60
40
20
0
Before
1 DAI
3 DAI
T1 (EB-31)
T2 (EB-35)
T3 (EB-40)
T4 (EB-65)
T5 (EF-81)
T6 (Pf1)
T7 (Pf2)
T8 (PP)
T9 (BM)
T10 (Control)

T_9 (BM) before inoculation. At one DAI, all the endophytic isolates and the reference cultures recorded per cent increase in activity by more than 37 over control with the maximum of 77.2 per cent increase in T_6 (Pf_1) followed by 76.4 in T_5 (EF-81) and 66.4 per cent increase in T_1 (EB-31). Nevertheless, the plants treated with the chemicals T_8 (PP) and T_9 (BM) showed an increase over control of 7.2 and 1.4 per cent respectively at one DAI. From one DAI to three DAI, the activity of the enzyme declined but it was more compared to control with all the treatments except T_9 (BM). At three DAI, the maximum activity was recorded in plants treated with T_6 (Pf_1) which had 83.8 per cent more activity than control followed by T_5 (EF-81) with 74.7 per cent and T_1 (EB-31) with 57.7 per cent increase in activity over control.

4.7.5 Isozyme analysis

4.7.5.1 Peroxidase (PO)

Native gel electrophoretic separation of the enzyme extract of leaves of seedlings, which received different treatments, after challenge inoculation expressed six isoforms of PO, designated as PO-1, PO-2, PO-3, PO-4, PO-5 and PO-6 (Plate 4.15). Plants treated with T_1 (EB-31) had PO-1, PO-2, PO-3 and PO-6. T_2 (EB-35) had PO-1, PO-3 and PO-6. Whereas the band for PO-1 was absent in all the remaining treatments *viz.*, T_3(EB-31) to T_{10} (Control). Similarly T_6 (Pf_1) and T_7 (Pf_2) had isoforms PO-2, PO-3, PO-5 and PO-6. All the other treatments *viz.*, T_3(EB-40),T_4(EB-65), T_5(EF-81), T_8(PP), T_9(BM), and T_{10}(Control) showed only three isoforms of PO.

4.7.5.2 Polyphenol oxidase (PPO)

Native PAGE electrophoretic separation of PPO isoforms of enzyme extract from treated leaves of cocoa seedlings showed seven isoforms of PPO *viz.*, PPO-1 to PPO-7 (Plate 4.16). The expression of isoforms varied among the different treatments. Bands for PPO-3, PPO-4, PPO-5, PPO-6 and PPO-7 were present in T_1 (EB-31) while T_2 (EB-35) had bands for only four isoforms *viz.*, PPO-2, PPO-3, PPO-4 and PPO-5 and T_3 (EB-40) had only PPO-2 and PPO-3. Leaves from treatment T_4 (EB-65) expressed five isoforms PPO-2, PPO-3, PPO-4, PPO-5 and PPO-6. T_5(EF-81) T_8 (PP) and T_9 (BM) showed similar banding pattern *viz.*, PPO-2, , PPO-3 and

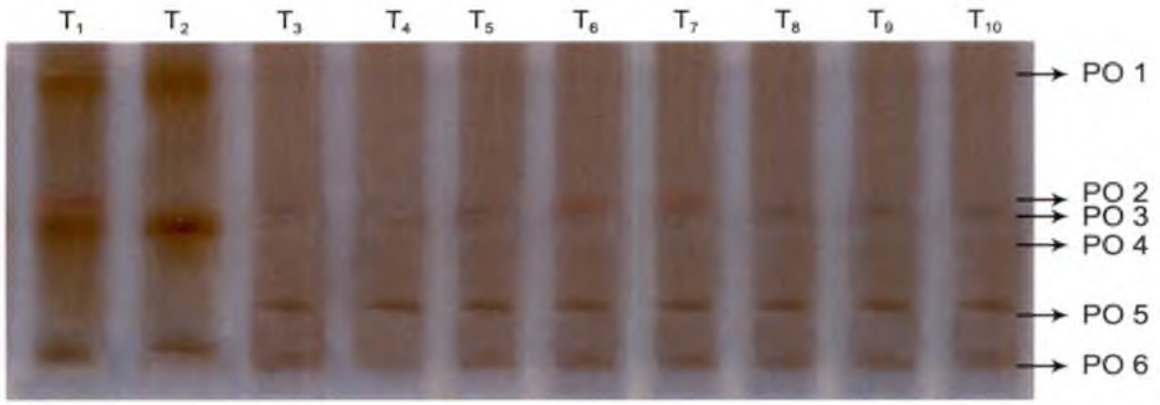

Plate 4.15 Native PAGE profile of peroxidase isoforms induced in response to challenge inoculation

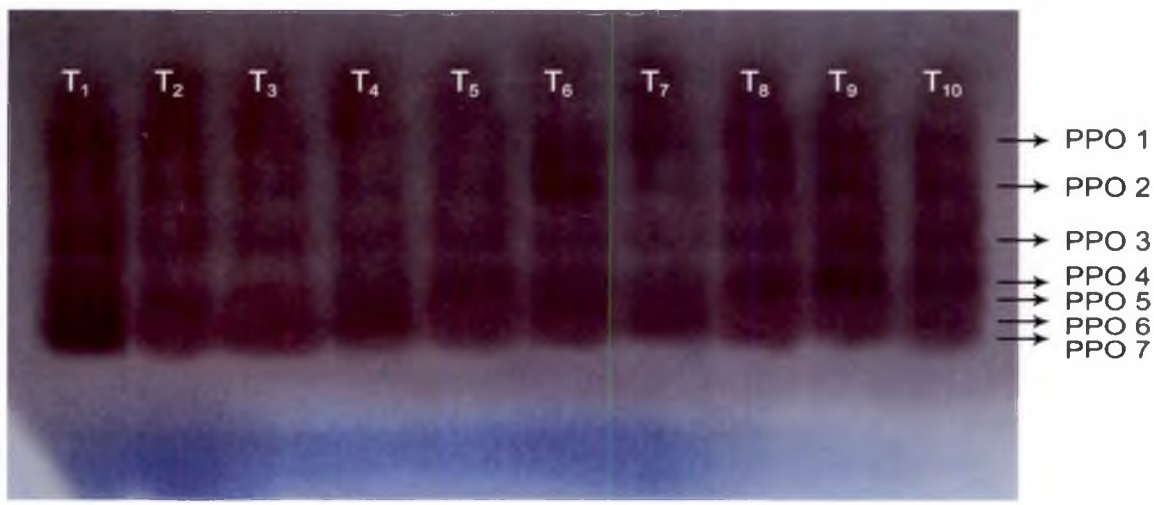

Plate 4.16 Native PAGE profile of polyphenol oxidase isoforms induced in response to challenge inoculation

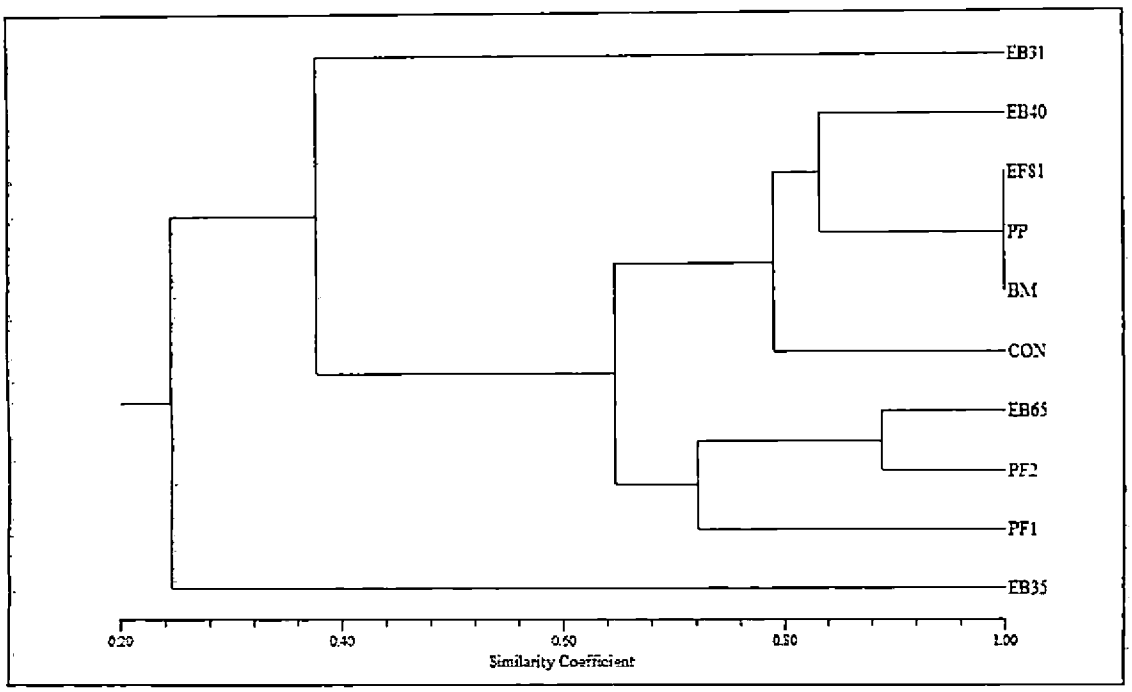

Fig. 4.15 Dendrogram showing linkage between different treatments on isoforms of peroxidase (PO) and polyphenol oxidase (PPO) of cocoa after challenge inoculation

Table 4.36 Cluster analysis of the dendrogram showing linkage between different treatments on isoforms of peroxidase and polyphenol oxidase of cocoa after challenge inoculation

Sl. No.	Cluster	Treatments
1.	Cluster I	T_1 (EB-31)
2.	Cluster II	T_3 (EB-40), T_5 (EF-81), T_8 (PP), T_9 (BM)
3.	Cluster III	T_{10} (Control)
4.	Cluster IV	T_4 (EB-65), T_7 (Pf2)
5.	Cluster V	T_6 (Pf1)
6.	Cluster VI	T_2 (EB-35)

PPO-4. While T_6 (Pf_1) had four isoforms , they are PPO-1, PPO-2, PPO-3 and PPO-5 and (Pf_2)had all the bands except the band for PPO-7.

4.7.5.3 Cluster analysis

The banding patterns of isoforms of the two enzymes (PO and PPO) of 10 treatments including the promising endophytes were subjected to cluster analysis (Fig. 4.15). The dendrogram indicated that, the treatments show variation with regard to the isozyme profiles of PO and PPO. At 80 per cent similarity index, the treatments belonged to six clusters and no sub clusters (Table 4.36). Further from the figure, it is evident that, T_1 (EB-31), T_{10} (control), T_6 (Pf_1) and T_2 (EB-35), are distinct, and are different from all the other treatments since they form separate clusters by their own which are cluster I, cluster III, cluster V and cluster VI respectively. While in the second cluster, T_5 (EF-81), T_8 (PP) and T_9 (BM) were 100 per cent similar and to these, T_3 (EB-40) showed 85 per cent similarity. The cluster IV consisted of T_4 (EB-65) and T_7 (Pf_2) which share 95 per cent similarity.

4.8 Field evaluation of selected promising endophytes against *Phytophthora* pod rot of cocoa

The efficacy of potential endophytes against *Phytophthora* pod rot in the field was studied in comparison with reference cultures and commonly used PP chemicals. The experiment was conducted in the existing cocoa garden at CCRP farm. The treatments were applied as described in the Materials and Methods. Observations on PPR incidence were recorded at weekly intervals and the per cent efficiency over control was calculated. Initial appearance of the disease was noticed in the experimental field during June second week with the onset of monsoon and then the first spraying was done. As the monsoon progressed, the disease incidence also showed an increasing trend till the end of August (Fig. 4.16). Thereafter it decreased with recession in the rain. The efficacy of the treatments varied during the three phases of the experiment *viz.*, after first, second and third spraying.

Results of the experiment for the initial phase *viz.*, after the first spraying are presented in Table 4.37. On the day of first spraying, disease incidence (less than one

per cent) was noticed in T_6 (Pf_1) and T_2 (EB-35). The disease appeared in all the treatments except T_1 (EB-31)and T_3 (EB-40) at one week after first spraying and the treatments showed a significant difference. Among the other treatments, the minimum incidence was noticed in T_4 (EB-65) followed by T_5 (EF-81) and T_9 (BM) and these were on par. The disease was maximum in T_8 (PP) followed by T_{10} (Control). During the second week after first spraying also there was significant difference among the various treatments with no disease incidence in T_1 (EB-31). Among others, the disease was the minimum in T_4 (EB-65) followed by T_9 (BM), T_2 (EB-35) and T_5 (EF-81). During this week also the maximum disease was in T_8 followed by T_{10} (Control). After three weeks of first spraying, the disease was observed in all the treatments with significant difference among them. As in week two, T_4 (EB-65) recorded the minimum disease followed by T_1 (EB-31), T_3(EB-40) and T_5(EF-81) and these were on par. At the end of three weeks, all the treatments except T_7 (Pf_2) had positive effect over control in reducing the disease. The maximum efficiency in reducing the disease was shown by T_4 (EB-65) and T_5 (EF-81) at three weeks after first spraying. However, in general the disease showed an increasing trend during this phase of the experiment.

Data on the disease incidence during the second phase *viz.,* the period from second spraying to third spraying are furnished in Table 4.38. Disease incidence on the day of second spraying showed significant difference among the treatments. The minimum per cent disease incidence was recorded in T_1 (EB-31) (6.2) followed by T_5 (EF-81) T_2 (EB-35), T_4 (EB-65) and T_3 (EB-40) during the week and these were on par. The treatment T_7 (28.4) recorded the maximum incidence. In the first week after second spraying, disease incidence was the least in T_2(EB-35) followed by T_1(EB-31), T_3(EB-40), T_4(EB-65) and T_5(EF-81) and these were also on par. The maximum disease was in T_8 (PP) followed by T_7 (Pf_2) and T_{10} (control). During the second week after second spraying, T_1 (EB-31) recorded the minimum per cent incidence of 9.4. It was followed by T_2 (EB-35), having 16.7 per cent disease incidence, T_4 (EB-65) and T_6 (Pf_1) which were on par. The disease was the maximum in T_8 (PP) followed by T_{10} (control) which were on par. In the third week after third spraying also the least disease incidence was in

T_1 (EB-31) which was followed by T_2 (EB-35), T_3 (EB-40) and T_5 (EF-81). During the week, the maximum disease incidence was noticed in control. During this phase of the experiment, the disease was on an increasing trend. All the treatments had a positive effect in reducing the disease incidence with maximum in T_1(EB-31)(63.4) and the minimum in T_7(Pf_2) and T_8 (PP)(4.1 per cent).

Results during the third phase of the field experiment are presented in Table 4.39. On the day of third spraying, the disease incidence differed significantly among the treatments with the minimum in T_6 (Pf_1) followed by T_1(EB-31), T_2(EB-35) and T_3(EB-40). From the last week in the second phase to the day of third spraying, the disease showed a declining trend in treatments T_1 (EB-31), T_2 (EB-35), T_3 (EB-40), T_4 (EB-65), T_5 (EF-81), T_7 (Pf_2) and T_8 (PP). The decline was most evident in T_6 (Pf_1). In which the disease came down from 30.2 per cent in eighth week, to 6.6 per cent which was the minimum during the period. The maximum disease incidence on the day of third spraying was in control. From the day of third spraying to first week after it, the disease declined in all the treatments except in control. During the week, the minimum disease was in T_6 (Pf_1) followed by T_7 (Pf_2), T_1(EB-31), T_3(EB-40) and T_2(EB-35) which were on par. The maximum was in T_{10} (control) and all the treatments except T_5(EF-81) were superior to the control during the first week after third spraying. In the second week after third spraying, the disease was the minimum in T_6 (Pf_1) which was closely followed by T_2(EB-35) and T_7 (Pf_2). The disease incidence was the maximum in T_{10} (control). However, from second to third week after third spraying, the disease declined in all the treatments including the control. By the last week of the third phase of the experiment, the incidence was nil in treatments T_1 (EB 31) to T_7 (Pf_2) but there was disease in treatments T_8(PP) and T_9(BM) in addition to the control. However the disease was significantly less in these treatments compared to control. It evident from the data presented in Tables 4.37 to 4.39 and from the Fig.4.16, that EB-31 followed by EB-35 maintained lowest level of the disease during the experiment. During the last phase of the experiment, treatments Pf_1 (90.3 per cent) followed by T_2(EB-35), T_7(Pf_2), T_3(EB-40) showed maximum efficiency in reducing the disease.

Table 4.37 Effect of different treatments on *Phytophthora* pod rot incidence in the field after first spraying

Sl. No.	Treatment	Per cent incidence of *Phytophthora* pod rot in the field						
		On the day of spraying	1 week after spraying	Per cent efficiency over control	2 week after spraying	Per cent efficiency over control	3 week after spraying	Per cent efficiency over control
1.	T_1 (EB-31)	0* (0.71)	0^{a} (0.71)**	100	0 (0.71)	100	3.3^{a} (1.76)	87.5
2.	T_2 (EB-35)	0.5 (1.21)	1.6^{ab} (1.37)	88.8	6.6^{ab} (2.63)	64.7	7.7^{ab} (2.73)	70.9
3.	T_3 (EB-40)	0 (0.71)	0^{a} (0.71)	100	3.7^{ab} (1.08)	80.2	3.7^{a} (1.80)	86.0
4.	T_4 (EB-65)	0 (0.71)	0.6^{a} (0.99)	95.8	2.0^{a} (1.46)	89.3	1.3^{a} (1.29)	95.1
5.	T_5 (EF-81)	0 (0.71)	1.2^{a} (1.15)	91.7	6.4^{abc} (2.63)	65.8	3.7^{a} (1.87)	86.0
6.	T_6 (Pf_1)	0.3 (1.05)	3.4^{ab} (1.79)	76.4	8.7^{bcd} (3.00)	53.5	18.6^{bc} (4.09)	29.8
7.	T_7 (Pf_2)	0 (0.71)	2.3^{ab} (1.55)	84.0	12.9^{cde} (3.62)	31.0	32.7^{c} (5.59)	-23.4
8.	T_8 (PP)	0 (0.71)	15.6^{c} (3.95)	- 8.3	21.4^{e} (4.68)	-14.4	22.1^{bc} (4.74)	16.6
9.	T_9 (BM)	0 (0.71)	1.3^{a} (1.16)	90.9	6.2^{abc} (2.63)	66.8	16.7^{bc} (4.11)	36.9
10.	T_{10} (Control)	0 (0.71)	14.4^{bc} (3.36)		18.7^{de} (4.19)		26.5^{c} (5.09)	

*Not analysed as most of the values were zero

** Values followed by same super script are not significantly different, mean of three replications, values in parentheses are $\sqrt{x+0.5}$ transformed, EB-Endophytic bacterium, EF-Endophytic fungus, Pf_1-*P. fluorescens* (KAU), Pf_2-*P. fluorescens* (TNAU), PP-Potassium phosphonate

Table 4.38 Effect of different treatments on *Phytophthora* pod rot incidence in the field after second spraying

Sl. No.	Treatment	Per cent incidence of *Phytophthora* pod rot in the field							
		On the day of spraying	Per cent efficiency over control	1 week after spraying	Per cent efficiency over control	2 week after spraying	Per cent efficiency over control	3 week after spraying	Per cent efficiency over control
1.	T_1 (EB-31)	6.2 [a] (2.32)*	73.3	13.0 [a] (3.37)	55.0	9.4 [a] (2.81)	75.8	14.2 [a] (3.22)	63.4
2.	T_2 (EB-35)	10.4 [ab] (3.04)	55.2	12.6 [a] (3.47)	56.4	16.7 [ab] (4.05)	57.0	16.9 [ab] (4.02)	56.4
3.	T_3 (EB-40)	15.8 [ab] (3.98)	31.9	17.2 [ab] (4.14)	40.5	26.4 [bc] (5.01)	32.0	20.2 [ab] (4.39)	47.9
4.	T_4 (EB-65)	15.4 [ab] (3.89)	33.6	19.3 [ab] (4.34)	33.2	17.8 [abc] (4.18)	54.1	28.4 [ab] (5.33)	26.8
5.	T_5 (EF-81)	10.1 [ab] (3.18)	56.5	20.6 [ab] (4.34)	28.7	28.1 [bc] (5.33)	27.6	27.3 [ab] (5.15)	29.6
6.	T_6 (Pf_1)	21.8 [ab] (4.53)	6.0	23.2 [ab] (4.76)	19.7	24.1 [abc] (4.84)	37.8	30.2 [ab] (5.53)	22.2
7.	T_7 (Pf_2)	28.4 [c] (5.37)	-22.4	31.8 [ab] (5.66)	-10.0	37.2 [bc] (6.07)	4.1	37.2 [b] (6.07)	4.1
8.	T_8 (PP)	26.8 [c] (5.17)	-15.5	35.1 [b] (5.94)	-21.5	40.5 [c] (6.36)	-4.4	37.2 [b] (6.11)	4.1
9.	T_9 (BM)	25.8 [c] (5.12)	-11.2	26.0 [ab] (5.13)	10.0	28.9 [bc] (5.42)	15.2	32.9 [b] (5.78)	15.2
10.	T_{10} (Control)	23.2 [ab] (4.55)		28.9 [ab] (5.32)		38.8 [c] (6.25)		38.8 [b] (6.25)	

* Values followed by same super script are not significantly different, mean of three replications, values in parentheses are $\sqrt{x+0.5}$ transformed, EB-Endophytic bacterium, EF-Endophytic fungus, Pf_1-*P. fluorescens* (KAU), Pf_2-*P. fluorescens* (TNAU), PP-Potassium phosphonate

Table 4.39 Effect of different treatments on *Phytophthora* pod rot incidence in the field after third spraying

Sl. No.	Treatment	Per cent incidence of *Phytophthora* pod rot in the field						
		On the day of spraying	Per cent efficiency over control	1 week after spraying	Per cent efficiency over control	2 week after spraying	Per cent efficiency over control	3 week after spraying
1.	T_1 (EB 31)	8.1 [a] (2.59)*	79.9	5.4 [ab] (2.19)	87.0	3.8 (1.83)	87.2	0 (0.71)
2.	T_2 (EB 35)	12.4 [a] (3.07)	69.2	6.6 [a] (1.97)	84.2	3.1 [a] (1.52)	89.6	0 (0.71)
3.	T_3 (EB 40)	13.5 [ab] (3.26)	66.4	6.2 [ab] (2.31)	85.1	3.4 [a] (1.56)	88.6	0 (0.71)
4.	T_4 (EB 65)	27.2 [abc] (5.07)	32.3	11.6 [ab] (3.35)	72.2	7.1 [a] (2.02)	76.1	0 (0.71)
5.	T_5 (EF 81)	22.9 [abc] (4.76)	43.0	20.9 [bc] (4.50)	50.0	12.0 [ab] (3.42)	59.7	0 (0.71)
6.	T_6 (Pf_1)	6.6 [a] (2.66)	83.6	4.1 [a] (1.96)	90.1	2.9 [a] (1.70)	90.3	0 (0.71)
7.	T_7 (Pf_2)	14.9 [a] (3.92)	62.9	4.7 (2.24)	88.8	3.1 [a] (1.70)	89.6	0 (0.71)
8.	T_8 (PP)	22.3 [abc] (4.76)	44.5	13.1 [ab] (3.52)	68.7	3.5 [a] (1.98)	88.2	1.3 [a] (1.28)
9.	T_9 (BM)	33.8 [bc] (5.83)	15.9	16.5 [ab] (4.08)	60.5	19. 7 [b] (4.49)	33.8	4.4 [ab] (2.14)
10.	T_{10} (Control)	40.2 [c] (6.36)		41.8 [c] (6.47)		29.78 [b] (5.47)		6.2 [c] (2.56)

* Values followed by same super script are not significantly different, mean of three replications, values in parentheses are $\sqrt{x+0.5}$ transformed, EB-Endophytic bacterium, EF-Endophytic fungus, Pf_1-*P. fluorescens* (KAU), Pf_2-*P. fluorescens* (TNAU), PP-Potassium phosphonate

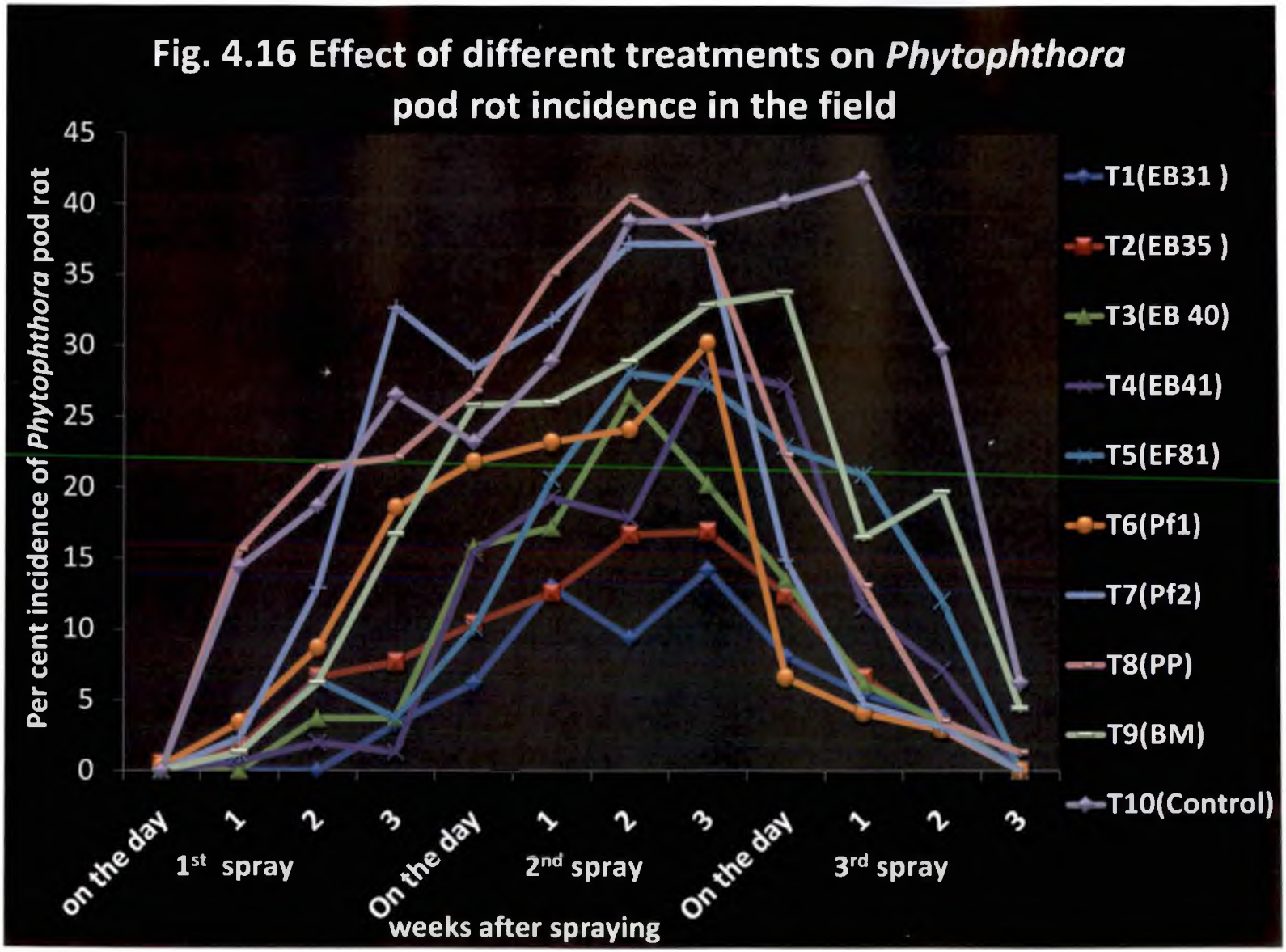
Fig. 4.16 Effect of different treatments on *Phytophthora* pod rot incidence in the field
Per cent incidence of *Phytophthora* pod rot
45
40
35
30
25
20
15
10
5
0
T1(EB31)
T2(EB35)
T3(EB 40)
T4(EB41)
T5(EF81)
T6(Pf1)
T7(Pf2)
T8(PP)
T9(BM)
T10(Control)
on the day
1
2
3
On the day
1
2
3
On the day
1
2
3
1st spray
2nd spray
3rd spray
weeks after spraying

4.9 Characterization of promising endophytes

The characters of the promising endophytes including one fungal isolate and four bacterial isolates were studied using standard protocols.

4.9.1 Biochemical characterization of promising bacterial endophytes

Response of the promising bacterial endophytes to various biochemical tests are presented in Table 4.40. Out of the four promising bacterial endophytes, three EB-31, EB-40 and EB-65 were gram negative and the these produced large colonies on KBA. The colonies of EB-40 were fluidal, flat, and opaque with undulate margin, while EB-31 and EB-65 had slimy, convex and translucent colonies with entire margin. The isolate EB-35 has gram positive rod shaped cells which formed large, dull, fluidal, flat, opaque and cream coloured colonies with undulate margin. The isolate produced pink coloured, non water soluble and non fluorescent pigment on KBA.These isolates produced water soluble fluorescent pigments and all were positive for arginine hydrolase and oxidase. EB-65 was positive for denitrification while, the other three were negative. Likewise, EB-31 and EB-35 hydrolyzed gelatin whereas EB-40 and EB-65 did not. One, *viz.*, EB-35 of these four bacterial endophytes hydrolyzed starch and produced H_2S. Except EB-65, the other three were catalase positive. EB-65 and EB-40 were urease positive and EB-35 was negative. However, EB-31 showed variable reaction towards the test. Similarly, EB-40 was positive for ornithine decarboxylase and lysine decarboxylase while the other three were negative. All the isolates utilized glucose, and citrate but not mannose, dulcitol, meso-inositol and sorbitol. Arabinose was utilized by EB-35 and EB-40 but not by the other two isolates. At the same time, EB-65, EB-35 and EB-31 utilized lactose but EB-40 did not. EB-35 showed positive reaction for acid formation test. The isolate utilized glucose, arabinose, mannitol, and citrate and did not form gas from glucose. Further, it hydrolyzed starch, casein, and gelatin. It reduced nitrate but was negative for phenylalamine deamination test, and positive for H_2S production. Based on cultural, morphological and biochemical properties, EB-35 was identified as *Bacillus*

Table 4.40 Cultural, morphological and biochemical characters of promising bacterial isolates

Sl. No.	Cultural/ morphological/ biochemical test	Reaction by isolates					
		EB-31	EB-35	EB-40	EB-65	Pf_1	Pf_2
1.	Grams staining	-	+	-	-	-	-
2.	Configuration	rod	rod	rod	rod	rod	rod
3.	Surface	large	large	large	large	large	large
4.	Sheen	rough& shining	dull	shining	smooth & shining	smooth & shining	rough & shining
5.	Fluidal/slimy	slimy	fluidal	fluidal	slimy	fluidal	slimy
6.	Elevation	convex	flat	flat	convex	convex	raised
7.	Margin	entire	undulate	undulate	entire	entire	entire
8.	Density	opaque	opaque	translucent	translucent	translucent	translucent
9.	Colour	cream	cream	shiny cream	yellowish green	yelloeish green	greenish yellow
10.	Pigments	WS	NWS	WS	WS	WS	WS
11.	Fluorescence(UV)	+	-	+	+	+	+
12.	Endospore	-	+	-	-	-	-
13.	Pyocyanin production	+	-	-	+	-	-
14.	Oxidase	+	-	+	+	+	+
15.	Catalase	+	+	+	-	+	+
16.	Arginine dihydrolase	+	-	+	+	+	+
17.	Lipase	-	d	+	+	-	+
18.	Levan formation from sucrose	-	-	-	-	-	-
19.	Gelatin liquefaction	+	d	-	+	d	+
20.	Starch hydrolysis	-	+	-	-	-	-
21.	Denitrification	-	-	-	+	-	-
22.	Citrate utilisation	+	+	+	+	+	+
23.	Lysine decarboxylase	-	-	+	-	-	d
24	Ornithine decarboxylase	-	-	+	-	-	+
24.	Urease	V	-	+	+	+	-
25.	Phenylalanine Deamination	-	-	-	-	-	-
26.	Nitrate reduction	V	+	-	-	+	+

Table 4.40 Continued.....

Sl. No.	Cultural/ morphological/ biochemical test	Reaction by isolates					
		EB-31	EB-35	EB-40	EB-65	Pf1	Pf2
27.	H_2S production	-	+	-	-	+	-
	Utilization of sugars						
28.	Glucose	+	+	+	+	+	+
29.	Sorbitol	-	-	-	-	-	-
30.	Dulcitol-	-	-	-	-	-	-
31.	Meso-inositol	-	-	-	-	-	-
32.	Mannose	-	-	-	-	-	-
33.	Fructose	+	+	+	+	+	+
34.	Sucrose	+	+	-	+	-	-
35.	Lactose	+	+	-	+	-	-
36.	Adonitol	d	-	-	d	-	-
37.	Cellobiose	-	-	-	-	-	-
38.	Arabinose	-	+	+	-	-	-

subtilis. Based on the aforesaid characters, EB-31 was tentatively identified as *Pseudomonas putida,* EB-40 as *Pseudomonas* sp. and EB-65 as *Pseudomonas aeruginosa*.

4.9.2 Identification of the promising endophytic fungal isolate

The fungal endophyte EF 81 was isolated on Martin's Rosebengal streptomycin agar from cocoa leaves collected from Palakkad (Table.4.5). It grows as raised colony on PDA, with entire margin; mycelium pale_yellow to pastel yellow but white at the margin. Conidiogenesis was abundant producing greyish green conidia which were oval to ellipsoidal (2.8-3.2×2.0-2.8 µm), smooth, borne on long, loose or irregular columns (Fig. 4.17). Based on the conidia and conidiophores characters, the fungus was identified as *Penicillium* sp. The isolate was later identified upto species level at the Indian Type Culture Collections (ITCC), Division of Mycology and Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi as *Penicillium minioluteum* (ID No.6905, Ref. No.A 81).

4.10 MOLECULAR CHARACTERIZATION OF PROMISING ENDOPHYTIC BACTERIAL ISOLATES

Genomic DNA of the promising endophytic bacterial isolates were isolated, purified and subjected to PCR for 16SrDNA amplification.

4.10.1 Isolation and purification of genomic DNA

The procedure as described in 3.10 was followed for bacterial DNA isolation. The quality of DNA isolated was tested using agarose gel electrophoresis. Good quality DNA was indicated by discrete bands (Plate 4.17).

4.10.2 PCR amplification

16SrDNA of the promising endophytic bacterial isolates were amplified using conserved eubacterial 16SrDNA primers (3.10.2). Analysis of PCR amplification was

carried out on 1.5 per cent agarose gel. Size of the amplified product was ≈1.6 kb (Plate 4.18).

4.10.3 Gel elution

Distinct bands obtained in PCR amplification were eluted and checked on 1.2 per cent agarose gel. Distinct bands with good concentration were observed similar to that obtained during PCR amplification thus indicating good recovery of fragments from the gel.

4.10.4 Cloning of the ligated product in *E. coli*

The ligated product was used to transform the competent cells prepared from *E. coli* culture using Fermentas kit (InsTAcloneTM PCR cloning kit #K1213). The transformants were selected using blue-white screening. The white colonies of the transformants were transferred to fresh medium. The 16SrDNA from promising endophytic bacterial isolates, cloned in *E. coli* cells were sent for sequencing at Macrogen, Korea.

4.10.5 Sequence data analysis

The sequence data obtained from Macrogen (Korea) are given in Annexure. The data were blasted in Ribosomal database project release 10 for sequence comparison and the interpretations are given here under.

The result obtained for sequence match for the four promising endophytic bacterial isolates are given in Tables 4.41 to 4.44. The match hit data shows similarity scores with sequences available in the database with which there is maximum similarity for the query sequence. The 16SrDNA sequence of EB-35 has shown a maximum similarity score of 0.900 with that of *Bacillus subtilis* subsp. *subtilis* (Table 4.41). Similarly the sequence of EB-40 showed maximum similarity score of 0.918 with that of *Pseudomonas plecoglossicida* (Table 4.42). However, in the case of EB-31(Table 4.43),

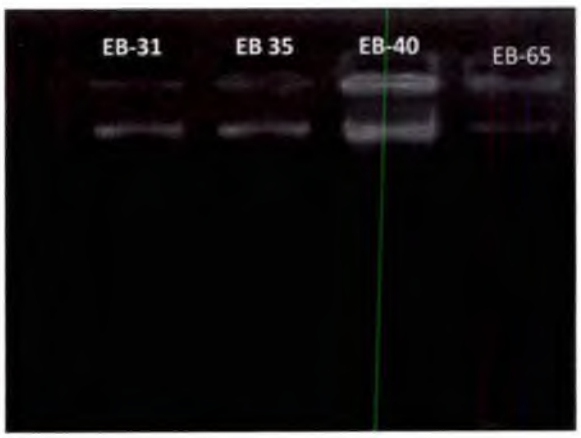

Plate 4.17 Agarose gel electrophoresis of genomic DNA of promising bacterial endophytes

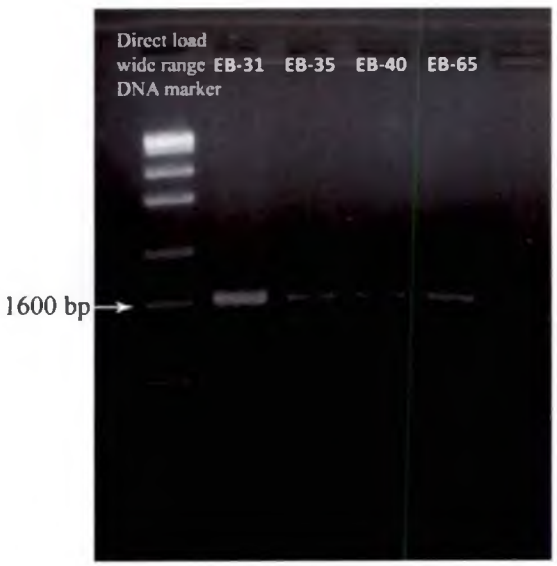

Plate 4.18 Agarose gel electrophoresis of PCR amplified 16SrDNA of promising bacterial endophytes

Table 4.41 Sequence match result for EB-35

Ribosomal Database Project: Release 10

Browsers | Classifier | LibCompare | SeqMatch | Probe Match | Tree Builder | Pyro | Taxomatic | seqCart | AssignGen
RDP Home | About | Announcements | Citation | Contacts | Resources | Related Sites | Tutorials

SeqMatch :: Result

[new match | help]

Seqmatch: version 3
RDP Data: release 10.17
Data Set: both type and non-type strains, both environmental (uncultured) sequences and isolates, near-full-length sequences (≥1200 bases), good quality sequences
Comments: 460821 sequences were included in the search
The screening was based on 7-base oligomers
Query Submit Date: Wed Dec 30 03:29:37 EST 2009
Match hit format: short ID, orientation, similarity score, S_ab score, unique common oligomers and sequence full name. More help is available.

Lineage:
Results for Query Sequence: unknown, 1329 unique oligos

no rank Root (20) (match sequences)
domain Bacteria (20)
phylum Firmicutes (20)
class "Bacilli" (20)
order Bacillales (20)
family Bacillaceae (20)
subfamily "Bacillaceae 1" (20)
supergenus Bacillus (20)
genus Bacillus a (20)

S000330830	not_calculated	0.900	1416	Bacillus subtilis subsp. subtilis; MO2; AY553095
S000330831	not_calculated	0.895	1408	Bacillus subtilis subsp. subtilis; MO3; AY553096
S000382420	not_calculated	0.895	1417	Bacillus subtilis subsp. subtilis; ATCC 6633; AB018486
S000391449	not_calculated	0.895	1443	Bacillus subtilis subsp. subtilis; N10; BGSC 3A17; AF318900
S000751666	not_calculated	0.898	1418	Bacillus subtilis subsp. subtilis; AU30; EF032678
S000751676	not_calculated	0.895	1420	Bacillus subtilis subsp. subtilis; AU25; EF032688
S000768732	not_calculated	0.895	1441	Bacillus subtilis subsp. subtilis; CCM 1999; ATCC 6633; DQ207730
S000825072	not_calculated	0.895	1383	Bacillus subtilis subsp. spizizenii; BCRC 17366; EF433402
S000870718	not_calculated	0.898	1390	Bacillus subtilis subsp. spizizenii; NBRC 101239; AB325584
S000891120	not_calculated	0.895	1404	Bacillus subtilis subsp. subtilis; GH54; AB301023
S000903166	not_calculated	0.900	1449	Bacillus subtilis subsp. subtilis; BS3902; EU047884
S000925396	not_calculated	0.896	1387	Bacillus sp. An11-1; AB244447
S001015682	not_calculated	0.895	1421	Bacillus sp. CPIS2; EU442609
S001096330	not_calculated	0.895	1435	Bacillus sp. zh161; EU526087
S001153004	not_calculated	0.898	1425	Bacillus sp. B8; EU362154
S001153006	not_calculated	0.897	1427	Bacillus sp. B10; EU362156
S001153014	not_calculated	0.900	1427	Bacillus sp. B18(2008); EU362164
S001153023	not_calculated	0.895	1426	Bacillus sp. B27(2008); EU362173
S001550542	not_calculated	0.898	1394	Bacillus subtilis subsp. spizizenii; R7; GQ122328
S001575466	not_calculated	0.896	1395	Bacillus subtilis subsp. subtilis; CRB115; GQ161967

Table 4.42 Sequence match result for EB-40

Ribosomal Database Project: Release 10

Browsers | Classifier | LibCompare | SeqMatch | Probe Match | Tree Builder | Pyro | Taxomatic | seqCart | AssignGen
RDP Home | About | Announcements | Citation | Contacts | Resources | Related Sites | Tutorials

SeqMatch :: Result

[new match | help]

Seqmatch: version 3
RDP Data: release 10.18
Data Set: both type and non-type strains, both environmental (uncultured) sequences and isolates, near-full-length sequences (≥1200 bases), good quality sequences
Comments: 469558 sequences were included in the search
The screening was based on 7-base oligomers
Query Submit Date: Thu Feb 04 23:50:00 EST 2010
Match hit format: short ID, orientation, similarity score, S_ab score, unique common oligomers and sequence full name. More help is available.

Lineage:
Results for Query Sequence: unknown, 539 unique oligos
no rank Root (20) (match sequences)
domain Bacteria (20)
phylum "Proteobacteria" (20)
class Gammaproteobacteria (20)
order Pseudomonadales (20)
family Pseudomonadaceae (20)
genus Pseudomonas (20)

S000538845	not_calculated	0.911	1349	Pseudomonas monteilii; R1; DQ071557
S000540545	not_calculated	0.881	1354	Pseudomonas putida; 1290; AY491973
S000559093	not_calculated	0.911	1326	Pseudomonas monteilii; R15; DQ095880
S000559095	not_calculated	0.913	1335	Pseudomonas plecoglossicida; R18; DQ095882
S000559096	not_calculated	0.918	1338	Pseudomonas plecoglossicida; R19; DQ095883
S000559097	not_calculated	0.883	1348	Pseudomonas plecoglossicida; R21; DQ095884
S000559100	not_calculated	0.918	1341	Pseudomonas plecoglossicida; R25; DQ095887
S000559113	not_calculated	0.881	1326	Pseudomonas plecoglossicida; S12; DQ095900
S000559120	not_calculated	0.881	1328	Pseudomonas plecoglossicida; S19; DQ095907
S000902410	not_calculated	0.885	1367	Pseudomonas plecoglossicida; CGMCC 2093; EF645247
S000959193	not_calculated	0.883	1270	uncultured bacterium; GXDC-26; EU250240
S000965096	not_calculated	0.905	1294	Pseudomonas sp. RD5PR2; AM911634
S000965097	not_calculated	0.918	1273	Pseudomonas sp. RD6SR1; AM911635
S000965102	not_calculated	0.911	1289	Pseudomonas sp. RD8MR3; AM911640
S000965103	not_calculated	0.911	1291	Pseudomonas sp. RD8PR2; AM911641
S000965104	not_calculated	0.911	1357	Pseudomonas sp. RD8PR3; AM911642
S000965105	not_calculated	0.911	1250	Pseudomonas sp. RD8SR1; AM911643
S000965108	not_calculated	0.918	1285	Pseudomonas sp. RD9SR1; AM911646
S000965132	not_calculated	0.911	1276	Pseudomonas sp. KLP2; AM911670
S001154015	not_calculated	0.911	1329	Pseudomonas putida; SR1156; EU826028

Table 4.43 Sequence match result for EB-31

Ribosomal Database Project: Release 10

Browsers | Classifier | LibCompare | SeqMatch | Probe Match | Tree Builder | Pyro | Taxomatic | seqCart | AssignGen
RDP Home | About | Announcements | Citation | Contacts | Resources | Related Sites | Tutorials

SeqMatch :: Result

[new match | help]

Seqmatch: version 3
RDP Data: release 10.13
Data Set: both type and non-type strains, both environmental (uncultured) sequences and isolates, near-full-length sequences (≥1200 bases), good quality sequences
Comments: 425489 sequences were included in the search
The screening was based on 7-base oligomers
Query Submit Date: Wed Aug 26 06:35:14 EDT 2009
Match hit format: short ID, orientation, similarity score, S_ab score, unique common oligomers and sequence full name. More help is available.

Lineage:
Results for Query Sequence: unknown, 1679 unique oligos

no rank Root (20) (match sequences)
domain Bacteria (20)
phylum Proteobacteria (20)
class Gammaproteobacteria (20)
order Pseudomonadales (20)
family Pseudomonadaceae (20)
genus Pseudomonas (20)

S000059454	not_calculated	0.617	1153	Pseudomonas putida; PB4071; AF180146
S000391033	not_calculated	0.627	1147	Pseudomonas putida; 5IIIASal; AF307864
S000391034	not_calculated	0.621	1141	Pseudomonas putida; 3IIIA2NH; AF307865
S000391035	not_calculated	0.625	1148	Pseudomonas putida; 3IA2NH; AF307866
S000391036	not_calculated	0.627	1146	Pseudomonas putida; PR1MN1; AF307867
S000396381	not_calculated	0.618	1150	Pseudomonas putida; RA2; AY121980
S000396382	not_calculated	0.620	1157	Pseudomonas putida; RA9; AY121981
S000396383	not_calculated	0.616	1168	Pseudomonas putida; RA16; AY121982
S000615564	not_calculated	0.606	1120	Pseudomonas putida; JH12; DQ232740
S000648424	not_calculated	0.601	1151	Pseudomonas monteilii; D5; DQ358076
S000965097	not_calculated	0.608	1273	Pseudomonas sp. RD6SR1; AM911635
S000965105	not_calculated	0.615	1250	Pseudomonas sp. RD8SR1; AM911643
S000965108	not_calculated	0.603	1285	Pseudomonas sp. RD9SR1; AM911646
S000965124	not_calculated	0.600	1229	Pseudomonas sp. RW7S1; AM911662
S000965132	not_calculated	0.604	1276	Pseudomonas sp. KLP2; AM911670
S000980083	not_calculated	0.604	1190	Pseudomonas sp. BF-6; EU289802
S000980226	not_calculated	0.604	1195	Pseudomonas mosselii; P5; EU301780
S001265228	not_calculated	0.601	1189	Pseudomonas sp. S7(2009); s7; FJ589738
S001417837	not_calculated	0.602	1235	Pseudomonas putida; AKM-P7; FJ897848
S001551016	not_calculated	0.602	1178	Pseudomonas stutzeri; GRFHAP-P14; GQ160905

Table 4.44 Sequence match result for EB-65

Ribosomal Database Project: Release 10

SeqMatch :: Result
[new match | help]

Seqmatch: version 3
RDP Data: release 10.13
Data Set: both type and non-type strains, both environmental (uncultured) sequences and isolates, near-full-length sequences (≥1200 bases), good quality sequences
Comments: 425489 sequences were included in the search
The screening was based on 7-base oligomers
Query Submit Date: Wed Aug 26 06:29:37 EDT 2009
Match hit format: short ID, orientation, similarity score, S_ab score, unique common oligomers and sequence full name. More help is available.

Lineage:

Results for Query Sequence: unknown, 945 unique oligos

no rank Root (20) (match sequences)
domain Bacteria (20)
phylum Proteobacteria (20)
class Gammaproteobacteria (20)
order Pseudomonadales (20)
family Pseudomonadaceae (20)
genus Pseudomonas (20)

S000386060	not_calculated	0.529	1458	Pseudomonas aeruginosa; WatG; AB117953
S000386395	not_calculated	0.529	1461	Pseudomonas aeruginosa PAO1; AE004949
S000458490	not_calculated	0.529	1460	Pseudomonas sp. pDL01; AF125317
S000497702	not_calculated	0.529	1461	Pseudomonas aeruginosa PAO1; AE004501
S000497705	not_calculated	0.529	1461	Pseudomonas aeruginosa PAO1; AE004844
S000497707	not_calculated	0.529	1461	Pseudomonas aeruginosa PAO1; AE004883
S000654054	not_calculated	0.529	1462	Pseudomonas aeruginosa; PAL106; DQ464061
S000654119	not_calculated	0.529	1433	Pseudomonas aeruginosa; PAL106; DQ466090
S000711168	not_calculated	0.529	1461	Pseudomonas aeruginosa PAO1; AE004091
S000711172	not_calculated	0.529	1462	Pseudomonas aeruginosa PAO1; AE004091
S000711174	not_calculated	0.529	1462	Pseudomonas aeruginosa PAO1; AE004091
S000711176	not_calculated	0.529	1462	Pseudomonas aeruginosa PAO1; AE004091
S000843854	not_calculated	0.529	1461	Pseudomonas aeruginosa; PT121; EF515832
S000927174	not_calculated	0.530	1466	Pseudomonas sp. J13; EU099379
S000957218	not_calculated	0.529	1466	uncultured bacterium; ZB1; EU236261
S000966737	not_calculated	0.529	1461	Pseudomonas aeruginosa; LP8; EU195558
S000980896	not_calculated	0.529	1462	Pseudomonas aeruginosa; MML2212; EU344794
S000981417	not_calculated	0.529	1494	Pseudomonas aeruginosa; MG-P13; EU364810
S001244356	not_calculated	0.529	1461	Pseudomonas aeruginosa LESB58; FM209186
S001418443	not_calculated	0.530	1462	Pseudomonas aeruginosa; WJ-1; FJ948174

the best score obtained was 0.627 with that of *Pseudomonas putida* and a score of 0.529 in the case of EB-65 (Table 4.44) with *Pseudomonas aeruginosa.*

4.11 Radiotracer study to test the entry and establishment of endophytes within the plant when applied externally

From among the promising endophytes, the four bacterial isolates were chosen for the radiotracer study, as bacteria could be easily multiplied and labeled. The bacteria were labeled using ^{32}P and applied on cocoa seedlings as explained in the Materials and Methods (Plate 4.19).

The autoradiograms revealed varied results *viz.*, the isolates EB-35 and EB-65 gave positive results when applied on leaves and pods whereas the results were negative for the other two isolates (EB-31 and EB-40). From the autoradiogram given as Plate 4.20 (A) it is clear that the radioactive bacteria have reached the conductive tissues of shoots and leaves which are situated above the treated leaf. The darker image of the lower leaf shows the tagged bacteria at the site of application. The images shown as Plate 4.20 (B) reveal the presence of labeled bacteria in the placenta of the pod.

Plate 4.19 Application of ^{32}P labeled promising bacterial endophytes on cocoa pods (A) and roots (B)

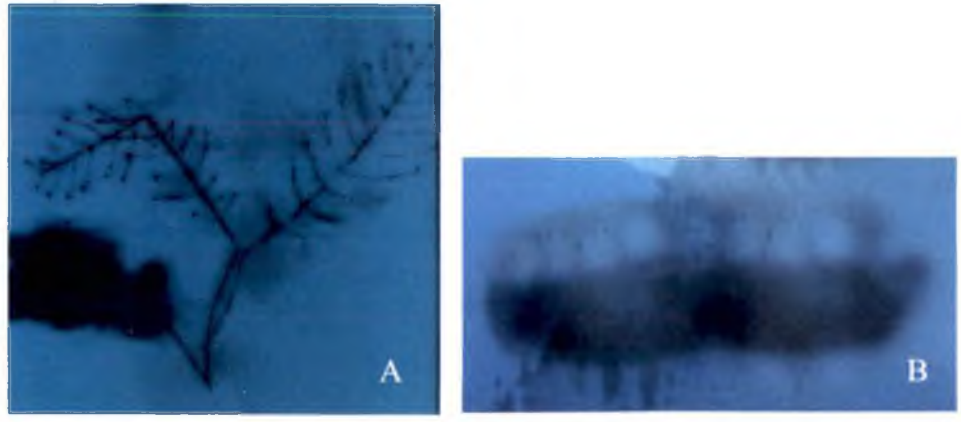

Plate 4.20 Autoradiogram showing ^{32}P labeled EB-35 in the conductive tissues of cocoa seedling (A) and EB-65 inside cocoa pod (B)

Discussion

5. DISCUSSION

The popularity of chocolate needs no reference. The demand for which is growing and it is doubtful whether the product from the plant, cocoa which prefer tropical rainforest condition will continue to meet it in the coming years. Moreover, several fungal diseases pose serious threat to the production and productivity of the crop. One of the major constraints faced by cocoa farmers in Kerala is the diseases which at times inflict heavy crop losses. The humid tropical climate of the State favours many fungal diseases, among which *Phytophthora* pod rot (PPR) caused by *Phytophthora palmivora* is the most severe one. This disease assumes serious proportions during monsoon periods, if proper disease management practices are not adopted regularly in time. Copper fungicides are widely used in the management of *Phytophthora* diseases, which results in the desired outturn only when integrated with cultural practices like shade regulation, pruning *etc.* But, often improper preparation of Bordeaux mixture and its untimely application failed to give the desired effects. Systemic fungicide metalaxyl and organophosphates like Fosetyl-Al and potassium phosphonate are also being used for the management of the disease. However, continuous use of chemical fungicides results in deleterious effects in the ecosystem. Hence, nowadays, attempts are being made to exploit the potential antagonistic microbes against the disease.

Many success stories have been reported on the biological management of *Phytophthora* diseases in which, the infected plant parts are in or near the soil. But limited success has been obtained in reducing the disease on aerial plant parts. This is due to the failure of establishment of biocontrol organisms on target points. This difficulty can be solved to some extent by the use of endophytes, which reside within the living tissues of the plant and hence are better protected from adverse environment as well as from the competition by native epiphytic and soil microflora. Studies conducted elsewhere revealed the usefulness of antagonistic endophytes in the biological management of diseases by inducing systemic resistance and in promoting plant growth (Chen *et al.*, 1995; Rubini *et al.*, 2005; Hallman *et al.*, 1997). Promising results for the control of diseases of cacao have been obtained using epiphytic mycoparasitic fungi and

bacteria (Krauss and Soberanis, 2001; Ten-Hoopen *et al.*, 2003; Bhavani, 2004; Tondje *et al.*,2006) and recently, many studies have revealed that some endophytic fungi restrict growth of pathogenic fungi of cocoa *in vitro* and reduce damage *in vivo* (Arnold *et al.*, 2003; Evans *et al.*, 2003; Holmes *et al.*, 2002, 2006; Rubini *et al.*, 2005; Tondje *et al.*, 2006), highlighting their status as a new source of biological control agents for combating cacao pathogens. According to Bhavani (2004), introduction of a well adapted effective antagonist into the cocoa garden will help in its multiplication and establishment and thereby lead to effective management of PPR of cocoa. Therefore, it will be an added advantage if such a native antagonist can exist as an endophyte. In spite of the fact that, the humid tropical climate of the state supports a vast diversity of epiphytic and endophytic microflora, not much work has been carried out for utilizing the efficacy of endophytes in the biocontrol of plant diseases in Kerala.

The intimate association of endophytes with plants makes them potential candidates for biological control. Rajendran *et al.* (2006) has opined that endophytic bacteria are an alternative to systemic pesticides that can be more reliable and ecologically as well as economically sustainable. Efficacy of endophytes for the management of fungal diseases of cocoa including black pod rot has already been reported (Arnold *et al.*, 2003; Mejia *et al*, 2008; Hanada *et al.*, 2008) so also for other oomycete diseases (Kim *et al.*, 2007).

Induction of systemic resistance in plants by beneficial microorganisms (ISR) is considered as an attractive alternative form of plant disease management, as it is based on extant resistance mechanisms in the plant and is effective against a broad spectrum of plant pathogens (van Loon *et al.*, 1997). In addition to induced systemic resistance endophytes are known to bring about disease suppression by various other modes of action also (Barka *et al.*, 2002; Bargabus *et al.*, 2002; Coombs *et al.*, 2004; Kloepper *et al.*, 2004 and Senthilkumar *et al.*, 2007). Hence it is pertinent to have a detailed investigation on the use of endophytes as a possible biological management strategy against the most serious disease of cocoa. Since different species of *Phytophthora* are attacking many crops in Kerala, this study will suggest a novel approach in the

biological management of other *Phytophthora* diseases also. In the present study, investigations were made on (1) diversity and distribution of endophytic microbes in cocoa grown at various locations in Kerala (2) potential of endophytes in the biological control of *Phytophthora* pod rot of cocoa, (3) mechanisms underlying the beneficial effects of endophytes on cocoa and finally (4) characterization and identification of promising endophytes obtained from cocoa.

5.1. ISOLATION AND IDENTIFICATION OF THE PATHOGEN

The present investigation was initiated by isolation of the pathogen from infected cocoa pods, which yielded a fungus with white mycelial growth on carrot agar. The pathogenicity of the isolate was established by inoculation on healthy pods. In India, though *P. palmivora* is the major causal agent, of PPR, *P. capsici* and *P. citrophthora* (Chowdappa *et al.*, 1993; Chowdappa and Chandramohanan, 1996) were also found in association with it. So, the cultural and morphological characters of the pathogen isolated were studied. The mycelium of the isolate was branched, hyaline and coenocytic. Sporangiophores developed from somatic hyphae were indeterminate and measured 46-129 μm in length. Sporangia were spherical when young, with less dense protoplasm. Mature sporangia were typically pear shaped with small but prominent papilla. The sporangia were borne terminally on the sporangiophore in a simple, sympodial fashion and were caducous. Deciduous sporangia had short and thick pedicel with an average L/B ratio of 1.15. Based on the cultural and morphological characters, which were typical as observed by earlier workers (Waterhouse, 1974; Waterhouse *et al.*, 1983; Brasier and Griffin, 1979; Zentmyer, 1988, Prem, 1995, Bhavani, 2004) and also based on the pathogenicty on cocoa, the isolate was identified as *Phytophthora palmivora* Butler (Butler).

5.2. ISOLATION AND ENUMERATION OF ENDOPHYTIC MICROFLORA FROM COCOA

The peculiar microclimate in cocoa gardens with high relative humidity favour growth of microorganisms *viz.*, epiphytes, endophytes and pathogens. Intending to study the diversity and distribution of endophytes in cocoa, plant samples were collected from

different locations of major cocoa growing areas of the State. Isolation and identification of endophytic microorganisms require elimination of surface microflora. So, usually the first step is surface sterilization followed by transfer of plant segments to appropriate culture medium. According to the crop, plant part used or research objective, modifications in the basic procedure may be adopted (Araujo *et al*, 2002). Different kinds of surface sterilization methods have been used by various workers (Bell *et al.*, 1995; Fisher *et al*; 1992; Mclnroy and Kloepper, 1995; and Shishidho *et al.*, 1995). In this study, it was found that sterilization using two per cent sodium hypochlorite solution for 10 min yielded good number of colonies with no growth in the sterility check, so, it was used for isolation of endophytes from samples collected from different locations. Trituration technique is considered as ideal for isolation of endophytes as it allows endophytic bacteria to be selectively isolated from vascular tissue in consistently high numbers (Hallmann *et al.*, 1997). Hence, this technique was used for isolation of endophytes from samples collected from various sites. Through this procedure, endophytes could be isolated in large numbers from different parts of the plant. By definition, endophytic microorganisms are those that inhabit the interior of plants, especially leaves, branches, stems and roots showing no apparent harm to the host. Moreover, according to Hallmann *et al.* (1997) total number of endophytes present at any time in a plant is controlled by the host and environment. Hence, for this study, endophytes were isolated from different parts of the cocoa plant *viz.*, leaves, shoots, pods and roots of healthy cocoa plants growing in different locations of major cocoa growing areas of the state. Quantitative estimation revealed that population of endophytes varied among different locations and plant parts studied. Accordingly, while fungi, bacteria and fluorescent pseudomonads were more in roots, yeasts were more in pods. Further, when fungi were more in samples from Palakkad, bacteria and fluorescent pseudomonads were more in that of Thrissur and yeasts in Kottayam. However, there was abundance of bacteria especially fluorescent pseudomonads among the microflora present within cocoa plants grown in Kerala. This is in contrary to many earlier reports in which fungal endophytes were chiefly isolated from cocoa (Rubini *et al.*,2005; Tondje *et al.*,2006). But recently, Melnick *et al.*, (2010) has pointed out the potentiality of endophytic

Bacillus sp. in the management of diseases of cocoa. Further they have also opined that use of bacterial endophytes in cocoa has been neglected by earlier workers. Since the endophytic population is influenced by external factors (Wilson and Carroll, 1994), collection from different parts and locations helped in the isolation of diverse group of organisms in sufficiently large numbers. There are reports saying that, from *Theobroma cacao* and *T. gileri* endophytes were isolated from, trunks (Evans *et al.*, 2003) and from leaves (Herre *et al.*, 2005; van Bael *et al.*, 2005).

As stated earlier, population of endophytes was more in the tender or feeder roots of cocoa than in other plant parts. Endophytes originate from rhizosphere or phyllosphere (Ryan *et al.*, 2008). They enter the endosphere mainly through natural openings. Since major share of microbial population of rhizosphere or phyllosphere is contributed by bacteria, it is reasonable to expect more bacteria in the endosphere also. Perusal of literature suggests that this is in agreement with the reports by other researchers (Mendes *et al.*, 2007, Shankar-Naik *et al.*, 2009) Moreover, identification of endophytes by many workers showed that, the major genera include common soil bacteria (Jacobs *et al.*, 1985; Leifort *et al.*, 1989; Berg *et al.*, 1994; Ferrador *et al.*, 2005; Rai *et al.*, 2007). This also explains why endophytic population is more in roots compared to other parts. Furthermore, according to Hallmann *et al.*, (1997) initial studies on the ecology of bacterial endophytes suggested that diversity among endophytes was similar to that in the rhizosphere, although total population densities were less within plants.

Similarly, in this study, isolation of endophytes was made on different media which helped in selecting appropriate colonies of each group of organism separately from large population. Endophytes have been isolated from practically all studied plants but, according to Lodge *et al.*, (1996), quantitative surveys of endophyte colonization patterns may be sensitive to leaf size, age, methodology, and growth medium. So, use of different media for isolation was helpful in getting more diverse group of organisms with varied nutrient requirements. Since preliminary isolations revealed predominance of bacteria, two media *viz.*, nutrient agar and Kings B agar were included for bacteria

and one medium each for others like, Martins rosebengal streptomycin agar for fungi, glucose yeast extract agar for yeasts and Kenknight's agar for actinomycetes. In the present study, endophytic fungi and yeasts were isolated from various parts of the plant though their population was less compared to bacteria, but no actinomycete could be isolated from cocoa. Lack of some specific nutrients in the medium used may be one of the reasons for this. However, there is an earlier report by Fisher *et al,* (1992) that, though several fungi and bacteria were present as endophytes, no actinomycete was isolated from maize. But, the present result is contradictory to report by Coombs *et al.*,(2004) who have isolated endophytic actinobacteria belonging to the genera *Streptomyces, Microbispora, Micromonospora*, from healthy cereal plants.

The population of yeasts was more than filamentous fungi in cocoa and they were present more in pods than other parts. In addition to the fungi, bacteria and actinomycetes, there are reports of significant numbers of endophytic yeasts present inside live plant tissues (Larran *et al.*, 2001; Larran *et al.*, 2002; Cao *et al.*, 2002; Maria and Sridhar, 2003; Tian *et al.*, 2004). Endophytic yeasts have been shown to promote maize growth under gnotobiotic and glasshouse conditions (Nassar *et al.*, 2005) and also epiphytic yeasts have been used for control of foliar diseases such as powdery mildews (Urquhart and Punja, 1997, 2002).

A total of 325 morphologically distinct colonies of endophytes obtained from different samples on different media were subcultured and purified for further use. These included isolates of 116 bacteria, 153 fluorescent Pseudomonads, 34 yeasts and 22 fungi.

5.3. *IN VITRO* ANTAGONISTIC EFFECT OF ENDOPHYTES AGAINST THE PATHOGEN

It is well known that *in vitro* results on antagonistic effects do not necessarily translate directly to what occurs in natural field conditions. Nonetheless, *in vitro* studies and their results are particularly useful for identifying likely candidates for biocontrol and for making intelligent guesses concerning the mechanisms by which they reduce pathogen damage (Mejia *et al.*, 2008). Several studies have shown that the interaction between plants and certain endophytic microorganisms was associated with beneficial

effects such as biological control of soil-borne fungal plant pathogens and plant growth promotion (Hallmann *et al.*, 1997; Sturz *et al.*, 2000; Narisawa *et al.*, 2004). On the other hand, many endophytic microorganisms have failed to show any beneficial effects on the inoculated host plant (Sturz *et al.*, 2000). Moreover, selection and identification of growth promoting and disease suppressive efficient antagonists through *in vitro* and *in vivo* assays are crucial for development of effective biocontrol strategy before launching field evaluation (Weller, 1988). Recent evidences suggest that endophytic fungi which restrict cacao pathogen growth *in vitro* are capable of reducing the damage *in vivo* also (Evans *et al.*, 2003; Holmes *et al.*, 2004, 2006; Tondje *et al.*, 2006). Hence in this study, endophytic isolates collected were screened for *in vitro* inhibitory effect against *Phytophthora*. In the preliminary screening, it was found that out of the 325 endophytic isolates collected from cocoa, 82 *viz.*, about 25 per cent of the total, were able to exert antagonism in varying degrees while the remaining were neutral. Similar line of work has been carried out by Sturz *et.al.*,(1998) who tested the endophytic flora from potato and clover which were grown as inter crop for antagonism towards *Rhizoctonia solani* and found that, of the bacteria tested, 74 per cent showed some degree of *in vitro* antibiosis to the clover and potato pathogen. There are reports of endophytic fungi isolated from healthy leaves and pods of cocoa which restricted *in vitro* growth of the three most common and economically important pathogens of cacao (*P. palmivora, M. roreri, and C. perniciosa*) (Arnold *et al.*, 2003; Rubini *et al.*, 2005). However, in the present study, endophytic bacteria were predominant among antagonists. Since bacteria were predominant in the endosphere, as found in the quantitative estimation, naturally they are represented more among the antagonists selected also. It was interesting to observe that, 28 per cent of the total bacteria, 19 per cent of the fluorescent pseudomonads, 62 per cent of yeasts and 18 per cent of the fungi were having varying levels of antagonistic properties towards the pathogen. Endophytic bacteria showing antagonism towards fungal pathogens have been studied in other woody plants (Trotel-Aziz, 2008) like pine (Ganley, *et al.*, 2008) and rubber (Philip, *et al.*, 2005). Melnick *et al.*, (2008) have suggested that natural endospore forming

endophytes may be better suited for long term colonization of cacao and could activate plant defense mechanisms more successfully than introduced fungal biocontrol agent.

Since the main objective of this study was to identify the most potential endophytes for management of PPR, the 82 antagonists were subjected to further evaluation to select efficient ones from among them. Thus, when tested individually, it was observed that, of these 82 antagonists, 44, registered more than 40 per cent inhibition of the pathogen and these were selected for subsequent screening on detached cocoa pods and leaves. It was found that, 64 per cent of the antagonistic bacteria, 76 per cent of the fluorescent pseudomonads, and all the four fungi gave more than 40 per cent inhibition while none of the yeasts was showing this level of inhibition.

5.4. EFFECT OF ANTAGONISTIC ENDOPHYTIC ISOLATES ON THE DISEASE ON DETACHED COCOA PODS AND LEAVES

Forty-four isolates, which registered more than 40 per cent *in vitro* inhibitory effect on the pathogen, were tested on detached pods. There was marked variation in the level of disease reduction on pods brought about by these isolates and based on this, 25 isolates showing greater efficiency *viz.*, those which recorded more than 55 per cent reduction in infection on pods were selected. Several endophytes exhibited dual antifungal mechanism through direct antagonism and through inducing plant defense reactions while some through either of the two (Trotel-Aziz *et al.*, 2008). Hence, those isolates possessing both antagonism and ability to induce systemic resistance may show greater efficiency in reducing the disease on pods. The results obtained are in line with reports by Bhavani, (2004) and Trotel-Aziz *et al.*, (2008).

When the 25 efficient isolates were again tested on detached leaves, it was noticed that, all of them showed more than 70 per cent effect in reducing the disease. This confirmed the result of the experiment on the pods. Further, it can be assumed that, the mechanisms of action by these microbes are more or less the same despite the change in the plant parts where applied. Other researchers have showed that leaf inoculations can be correlated with pod inoculations (Iwaro *et al* ., 1997a) and that results of leaf-disc tests show medium to high predictability levels on disease

suppression on pods (Iwaro *et al* ., 1997b; Tahi *et al.*,2000; Tahi *et al* ., 2006). So, all the 25 efficient antagonistic isolates were selected for evaluation of growth promoting ability.

These 25 efficient antagonists represent 7.8 per cent of total bacteria, an equal per cent of total fluorescent pseudomonads and 18 per cent of the fungi isolated. In cocoa, similar result was reported by Macagnan *et al.* (2006) who, based on the data from the bioassay on detached pods showed that only seven per cent of the epiphytic endospore-forming bacteria isolated from cocoa pods were able to inhibit the germination of *Crinipellis perniciosa* basidiospores at levels of 60 per cent to 100 per cent.

5.5. EVALUATION OF ENDOPHYTES FOR GROWTH PROMOTION IN COCOA

5.5.1 Effect on growth parameters

The intimate association between plants and microbes itself suggests that mutual benefits must be enjoyed by both the group. Moreover, most of the studies on endophytes have proved their prominent role as plant growth promoters (Chanway, 1996; Hallmann *et al.*, 1997; Nejad and Johnson, 2000; Muthukumaraswamy *et al.*, 2000; Barka *et al.*, 2002; Hong *et al.*, 2004). Further, it will be an additional benefit if the efficient antagonist which may be used as biocontrol agent possess growth promoting ability also. Hence in the present study the antagonistic endophytes selected based on efficient inhibition of the pathogen in dual culture and reduction of disease on detached pods and leaves were subjected to evaluation for growth promotion on cocoa seedlings. The methods of application of endophytes are similar to that of other beneficial microbes *viz.*, seed treatment, soil application or foliar spray. Inoculation of endophytes has been achieved by placing mycelia in coleoptile tissue (Latch and Christensen 1985), syringe inoculation (Leuchtmann and Clay 1988), and soaking seeds in spore suspensions in the case of grasses. Similarly, sprays of spore suspensions have been used to introduce endophytes into beans and barley (Boyle *et al.*, 2001). In another

study, *Bacillus subtilis* strain BS-2 was able to colonize, propagate and move in cabbage plants after inoculation to the plants by seed dipping, watering or leaf daubing (Hong, *et al.*, 2004). Areas of emerging lateral roots and germinating radicle also act as the way for entry of endophytic bacteria into plant system (Barraquio *et al.*,1997). Further endophyte population was more in the tender or feeder roots of cocoa than other plant parts as observed in the previous experiment in this study. This suggests their entry through roots. Hence the efficient antagonistic endophytes were applied as seed treatment, soil drenching and foliar application for evaluation of growth promoting effect. This experiment was done in both sterilized and non sterilized soil in order to see the effect of endophytes both in the presence and absence of native soil microflora. The results showed that, seed treatment with endophytes had a profound effect on germination of the beans. There was early as well as increased germination of beans sown in sterilized and non sterilized potting mixture. However, the effect was less prominent in the later. This result suggests that lack of competition from soil microflora, resulted in better activity of the beneficial endophytes leading to more effect on seed germination under sterile condition. Increased germination and enhanced growth and vigour of many plants due to seed coating with *Pseudomonas fluorescens* had been reported by Kumar (2002), Kumar *et al.* (2002), and Thomas and Vijayan (2003). Many authors suggested that most endophytes originate from rhizosphere or phyllosphere (Mendes *et al.* 2007) and they enter the endosphere mainly through natural openings and, some may be transmitted through the seed (Ryan *et al*, 2008).

Biometric observations recorded in the present study strongly indicated the role of endophytes as plant growth promoters. Positive effect of endophytes was noticed in most of the growth parameters studied. More than 28 per cent increase in seedling height was recorded by seed treatment by four isolates *viz.*, EB-31, EB-35, EB-40, EB-65. Similarly, upto 64 per cent increase in number of leaves was recorded by EB-31 followed by more than 20 per cent increase effected by five isolates. However four isolates had negative effect on number of leaves. With regard to girth at collar, except EB-1 all other isolates gave positive effect. In the case of fresh and dry weight of shoot,

however, out of the 25 isolates 11 and 18 isolates respectively recorded positive effect while the rest had negative effect. Although the interaction between host plants and endophytes are not fully understood, mechanisms such as nitrogen fixation (Lee *et al.*, 1999, Sevilla *et al.* 2001, Hurek *et al.* 2002 and Iniguez *et al.* 2004), production of growth promoting substances and increased resistance to pathogens had been suggested (Muthukumaraswamy *et al.*, 2002). Further, mechanisms by which endophytes enhance plant growth may be similar to those by which plant growth promoting rhizobacteria do. Growth promoting effect of PGPR is well documented which include production of phytohormones, inducing systemic resistance, protecting from plant pathogens, and enhancing availability of nutrients (Frommel *et al.*,1991).

Since the endophytic population originate from PGPR (Ryan *et al.*, 2008; Shankar-Naik *et. al.*, 2009), obviously, same effects may be expected from endophytic microbes also. Further, the results of the present study are in agreement with that reported earlier in cocoa in which, increased growth rate, reflected as higher fresh and dry weight, plant height, root length, root weight of cocoa seedlings as a result of endophytic colonization of *Trichoderma hamatum* has been observed by Bae *et al.* (2009). Similarly, Sturz *et al.* (1998) who studied 25 endophytic bacterial species from 18 genera found that, 21 per cent were plant growth promoting, 56 per cent were plant growth neutral and 24 per cent were plant growth inhibiting in potato. Rajan *et al.*, (2002) has reported enhanced tillering, and overall growth in ginger due to endophytes isolated from different ginger tracts. In another study, endophytic bacteria enhanced growth of sunflower seedlings (Forchetti *et al.*, 2010). Fu *et al.* (1999) also had reported that, when one isolate of endophytic bacteria promoted shoot growth in cotton, another had no effect on it.

Length of root and fresh and dry weight of roots were also influenced by endophyte treatment. Twenty out of 25 isolates augmented root length and fresh weight of roots, while 18 out of the 25 augmented dry weight of roots. This result also supports earlier reports of enhanced growth of roots of grapevine plantlets due to endophyte treatment (Barka *et al.*, 2002) and average increase of 70 per cent over control in root and shoot growth of corn and beans (Bacon and Hinton, 2002).

5.5.2 Selection of potential endophytes

It was noticed that, eight isolates *viz.*, EB-19, EB-22, EB-31, EB-35, EB-40, EB-41, EB-65 and EF-81 showed maximum growth promoting effect by augmenting seedling height, number of leaves, fresh and dry weight of shoot and girth at collar. They have shown more efficiency on enhancing the root parameters too. Further, from their effect on the disease on detached pods and leaves, it was observed that these isolates had better efficiency in reducing the disease also. These were selected as potential endophytes since they exhibited good antagonism coupled with good growth promoting ability.

The eight potential endophytes were collected from four districts of Kerala *viz.*, Idukki (EB-35 and EB-41), Pathanamthitta (EB-65), Thrissur (EB-19, EB-22, EB-31 and EB-40) and Palakkad (EF-81) further, it is interesting to note that these include five isolates of fluorescent pseudomonads, two bacteria and one fungus. Growth promoting and antagonistic ability of fluorescent pseudomonads is well documented as they form the major community among PGPR (Kloepper *et al.*, 1988; Glick,1995; Paulitz *et al.*, 1998; Raupach and Kloepper, 1998; Perez *et al.*, 2001; Vijayaraghavan, 2007).

The present result suggests that the major share of potential endophytes *viz.*, those having growth promoting ability and bio control efficiency is represented by fluorescent pseudomonads isolated from cocoa pods. Though the population was more in roots, when selection was made based on antagonistic ability ahead of growth promoting effect, the representation was restricted to pods and leaves. Since the pathogen attacks aerial plant parts, this finding indicates the co-evolution of antagonists and pathogens. Accordingly niche exclusion and host colonization are suggested as mechanisms of antagonism by endophytic bacteria (Cook and Baker, 1983; Backman *et al.*, (1997).

5.6 MECHANISMS OF ACTION OF ENDOPHYTES

The 25 efficient endophytes tested for growth promotion on cocoa seedlings were shortlisted to eight potential endophytes. These were subjected to various tests for

mechanisms of action. As already stated, endophytes originate predominantly from rhizosphere and phyllosphere (Ryan *et al.*, 2008). Hence it can be assumed that the attributes for a good PGPR may hold good for a good endophyte too. So, the potential endophytes selected were initially tested for various parameters included in the PGPR index (Samanta and Dutta, 2004) and a PGPE (Plant Growth Promoting Endophyte) index was worked out for each potential endophytic isolate. The objective of this scoring was to select better isolates from among the eight potential endophytes for testing under actual field conditions. Samanta and Dutta (2004) reported differences in PGPI among the isolates. According to them, characteristics of the organism like P solubilizing ability, ammonia, IAA and HCN production have significant role for a potential biocontrol agent. In order to arrive at the PGPE index, in addition to the aforementioned attributes antagonism index (based on dual culture study) and vigour index of the treated seedlings were also calculated. Chet (1990;1993) suggested the involvement of various compounds of microbial origin in growth promotion and disease suppression. Several researchers have studied production of these substances by endophytes (Nejad and Johnson, 2000; Sturz *et al.*, 2000; Sessitsch *et al.*, 2002; Kuklinsky-Sobral *et al.*, 2004; Forchetti *et al.*, 2007). Two reference cultures namely *Pseudomonas fluorescens* (KAU) (Pf_1) and *P. fluorescens* (TNAU) (Pf_2) were also subjected to the tests for mechanism of action for comparison.

5.6.1 Production of ammonia

The capacity to produce ammonia is an attribute which is directly related to antagonistic ability of a bio-control organism. In the present investigation, all the isolates tested produced ammonia. However, EB-35, EB-40, EF-81 and Pf_1 produced more while EB-22 and Pf_2 produced less ammonia. It is not sure whether the endophyte can produce volatiles like ammonia *in planta* (Ryu *et al.*, 2003). However, since endophytes originate from rhizosphere, and they are applied as seed treatment and soil application also, it is possible that ammonia production is one among the mechanisms by which endophytes show antagonistic and growth promoting effect. Samanta and Dutta

(2004) had already proved the role of ammonia production by PGPR from mustard in suppressing *S. sclerotiorum*.

5.6.2 Production of HCN

HCN is considered as a possible and perhaps frequent mechanism by which bacteria suppress plant pathogens (Ross and Ryder, 1994; Maurhofer *et al.*, 1994b). However, here it was noticed that all the isolates tested were unable to produce HCN. This result confirmed the earlier report by Nejad and Johnson (2000), who found that most of the endophyte isolates from oil seed rape were HCN negative. But they have suggested that volatiles other than HCN may be involved in the antagonism expressed by the endophytic isolates.

5.6.3 Phosphate solubilization

Phosphorus is an essential element for plants which they require in solution as phosphate anions such as HPO_4^{2-} and $H_2PO_4^-$. Being highly reactive, may be immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+} or may be adsorbed to Fe or Al-oxides and Al or Ca-carbonates. In these forms, P is highly insoluble and unavailable to plants. Microorganisms play a fundamental role in biogeochemical cycling of P in nature. It is a well established fact that ability of a beneficial microorganism to solubilize insoluble phosphorus present in soil and make it available for plants is directly related to its efficiency in plant growth promotion. When the potential isolates and reference cultures Pf_1 and Pf_2 were compared for their P solubilising ability, it was found that, all the bacterial isolates solubilized tricalcium phosphate. The maximum P solubilization was recorded by EB-35 followed by EB-31, Pf_2 and EB-40. From the data on growth parameters, it was observed that, these isolates had effected more than 28 per cent increase in seedling height, 20 per cent more leaves. They had augmented root parameters also.

Capacity of endophytic bacteria to solubilize phosphate has already been reported (Kuklinsky-Sobral *et al.*, 2004). Endophytic bacteria from sunflower also expressed P solubilising ability (Forchetti *et al.*, 2007). Fungi belonging to the genus

Penicillium are considered to be key group of soil microflora involved in P cycling (Whitelaw, 2000). The organic acids produced by these microbes can directly dissolve P precipitates or they chelate the cations that precipitate P with concomitant release of P into solution (Gadd, 1999). However, the endophytic fungal isolate EF-81 showed no P solubilizing ability. Though most of the species of *Penicillium* are P solubilizers, this particular strain may be unable to utilize tricalsium phosphate or the quantity of P brought into solution by this isolate may be too less to be detected by the method used. This isolate was identified as *P. minioluteum* and it was earlier reported to be unable to show clear zone on solid medium containing tricalsium phosphate and it solubilized very meager (14.5 mg l^{-1}) amount of P (Wakelin *et al.*, 2004). However, this isolate was able to produce IAA and ammonia which is reflected as the growth promoting and antagonistic properties.

5.6.4 IAA Production

Production of phytohormones as one of the mechanisms involved in growth promotion by endophytes has been suggested by Sturz *et al.* (2000) and Sessitsch *et al.* (2002). In the present study, the endophytic isolates produced growth promoting effect of cocoa which suggested their ability to produce phytohormones. Hence the potential isolates selected based on antagonistic and growth promoting ability were tested for the capacity to produce IAA. All the isolates, *viz.*, eight endophytes and two reference cultures produced varying levels of IAA, ranging from 7.5 to 56.8 μg ml^{-1}. The maximum quantity of IAA was produced by EB-35 followed by EB-40. The result is in agreement with the enhanced seedling height and other characters effected by the potential isolates in the nursery experiment. According to Mendes *et al.* (2007), IAA production is more prevalent among the bacterial endophytes than among rhizosphere bacteria of sugarcane further this trait was more frequent among bacteria from the stem. Based this, they have suggested that, plants select for endophytic bacteria with this trait or IAA-producing bacteria possess other traits that allow them to more effectively colonise the inner plant tissue. The fungal isolate, selected as potential endophyte in this study (EF-81) also produced IAA. The growth promoting effect produced by this isolate

is chiefly attributed to its capacity to produce IAA. Although the effect and role of IAA production by endophytic bacteria in growth promotion need to be investigated, this trait is considered to be one of the major mechanisms involved in growth promotion by the rhizosphere bacteria. However, several other workers also have reported IAA production by endophytic bacteria in different crops. Many of the endophytic bacteria isolated from the stems of sugarcane plants by Mendes *et al.*,(2007) were shown to produce IAA. Kuklinsky-Sobral *et al.*, (2004) reported IAA production by endophytic bacteria from soyabean plants. IAA production by most of the isolates from soybean had been reported by Hung, *et al.*, (2007) also.

5.6.5 Antagonism Index (AI)

The AI of the eight potential endophytic isolates was calculated based on the *in vitro* inhibition of *Phytophthora* in dual culture (Kasinathan,1998). The A1 for reference cultures was also worked out for comparison. The isolates showed marked difference with regard to the AI. A1 value above 2200 was recorded by EB-31 and EB-35. It was above 1800 for Pf_1, EF-81, Pf_2, EB-19, EB-22 and EB-40. The inhibitory response effected by these isolates may be due to the production of secondary metabolites and antifungal compounds (Maurhoffer *et al.*, 1998; Sessitsch *et al.*, 2004). The metabolites include Oomycin, A (Howie and Suslow, 1991), phenazine-1, carboxylic acid (PCA) (Delany *et al.*, 2001) 2,4-diacetyl phloroglucinol (2,4,-DAPG) (Mavrodi *et al.*, 2001), cyclic lipopetides (Raaijmakers *et al.*,2002), pyrrolnitrin (de Souza and Raaijmakers 2003) *etc.* produced by pseudomonads. *Bacillus subtilis* is a known producer of cyclic peptide antibiotics such as Iturin, Bacillomycin (Wilhelm *et al.*,1998) Various species of *Bacillus* produce surfactin, amphomycin, valinomycin *etc.* (Asoka and Shoda, 1996). Difference in inhibitory response produced by the isolates may be attributed to the difference in the inhibitory substance released by them. Bacon and Hinton (2002) suggested that not all endophytic strains produced the same inhibitory substance. Some isolates caused lysis of the pathogen up on contact with the hyphae, which resulted in lysis of the entire fungal colony (contact inhibition) while others produced a diffusible inhibitory substance into the medium that produced necrotic areas

in hyphae along the edge of the colony (Bacon and Hinton, 2002). The type of inhibition observed in the present study suggest the involvement of the later type of action by the isolates tested. Bacon and Hinton (2002) also suggested that, the variation in potency and type of antagonism exhibited by different strains of endophytic bacteria reflected the variation in either amount or type of inhibitory substances produced.

5.6.6 Vigour index (VI)

Being another parameter for calculating the PGPE index, the vigour index of cocoa seedlings treated with the eight potential endophytes and the reference cultures were calculated. Based on the mean shoot length, root length and per cent germination, of treated seedling, the growth promoting effect by a beneficial organism is directly depicted in the vigour index and hence it is crucial for selection of better isolates. The highest value for vigour index was recorded for seedlings treated with EB-35 followed by EB-31 and Pf_1. Isolates EB-22 and Ef-81 also improved the VI of cocoa seedlings. The role of endophytes as growth promoters was clearly indicted by the higher VI effected by these isolates. In addition to the mechanisms as that of PGPR, mechanisms such as N fixing ability, enhancing carbon sequestration potential (Taghavi, *et al.*,2009) also have been attributed to the growth promoting effect of potential of endophytes. The effect of endophytes on growth parameters studied was already discussed.

5.6.7 PGPE index

Data on the growth promoting parameters showed that the isolates differ in their performance regarding different parameters studied. However, some of them show superiority in many of the growth promoting attributes. Hence, data on 'vigour index', 'antagonism index', P solubilization, IAA, NH_3 and HCN production were converted into 1-4 scores and from this, the PGPE index, originally developed by Samanta and Dutta (2004) for comparing Plant Growth Promoting Rhizobacteria (PGPR index) was calculated for the potential endophytes. They were then ranked based on the PGPE index considering all the growth promoting parameters and antagonistic potential. It was found that EB-35 and EB-40 have got the maximum score in most of the parameters studied so

they had the higher values for PGPE index. This index provided a comprehensive measure of the aforesaid parameters which contributed to the efficiency of the isolate. These parameters are studied by many workers for comparing beneficial endophytes (Nejad and Johnson, 2000; Bacon and Hinton, 2002; Kuklinsky-Sobral *et al.*, 2004; Nassar *et al.*, 2005; Hung *et al.*, 2007; Forchetti *et al.*, 2007). The potential endophytic isolates were compared based on the PGPE index. Five among them *viz.*, EB-31, EB-35, EB-40, EB-65 and EF-81 recorded the index above 60 and therefore they were selected for further studies as promising endophytes.

5.6.8 Tests for the volatile and non volatile metabolites

Cook and Baker (1983) suggested different mechanisms by which the endophytic microbes controlled *Fusarium* wilt of different crops. These mechanisms include production of antifungal compounds, siderophore production, nutrient competition, niche exclusion and induction of systemic resistance. It is possible that, several of these mechanisms play a role in the biological control exhibited by these organisms. The elucidation of the mechanisms promoting plant growth and suppressing disease will help to select species and conditions that lead to greater plant benefits (Melnick *et al.*, 2008). Hence, the selected promising endophytes were subjected to further detailed studies on the mechanisms of action.

5.6.9 Production of volatile inhibitory metabolites

Since all of the eight potential endophytes were HCN negative, this test was coducted to know their ability to produce volatile compounds other than HCN. It was interesting to note that all the promising isolates exhibited positive response to this test. EB-35 produced the maximum amount of volatiles, followed by EB-31, EB-65 and Pf_2. It is hence concluded that all these isolates produced antifungal volatile substances other than HCN. Moreover, from 43.2 per cent (EF-81) to 59.2 per cent (EB-35) inhibition was effected by way of volatile inhibitory metabolites produced by these isolates. The results of this experiment are in agreement with the report by Nejad and Johnson (2000), who found that most of the endophytic isolates from oil seed rape were HCN negative

but the isolates produced other volatile metabolites with fungal inhibitory action. According to Ryu *et al.* (2003), the volatiles such as 2-3 butanediol and aecotin produced by bacteria are responsible for plant growth promotion. However, it is yet to be determined if volatiles could be produced within the plant system.

5.6.10 Production of diffusible non-volatile inhibitory metabolites

Observations on the inhibitory response in dual culture suggested that not all strains produced the same inhibitory effect. Hence this test was done to compare the promising isolates based on diffusible inhibitory metabolites. It was observed that all the isolates tested produced inhibitory substances which diffused into the medium through the cellophane and inhibited growth of *Phytophthora*. The isolates showed variation in the inhibition produced by this way too. The maximum inhibition was noticed in EB-35 and EB-40 (59.4) followed by EB-65 (58.3), EB-31 (54.4) and Pf_2 (51.7). The reason may be the involvement of production of various compounds of microbial origin as according to Chet (1990;1993). Bacon and Hinton (2002), also suggested that, some endophytic bacteria produced a diffusible inhibitory substance into the medium that produced necrotic areas in hyphae along the edge of a colony. The variation in potency and type of antagonism exhibited by different strains of endophytic bacteria reflected variation in either the amount or the types of inhibitory substances produced. These substances may be unstable or poorly diffused into the agar. Alternatively, each strain may have membranes that are differentially permeable to the inhibitor, thereby restricting its diffusion into the medium. Another possibility suggested is that, the fungus is inhibited by non-antibiotic mechanisms. However, the differences in appearance of hyphae of the pathogen are caused by either contact or diffusional inhibition. The results also suggest that there are probably more than one inhibitor produced by the strains (Bacon and Hinton, 2002).

5.6.11. Siderophore production

Bacterial siderophores are low-molecular-weight compounds with high Fe3+ chelating affinity (Sharma and Johri, 2003) responsible for the solubilization and

transport of this element into bacterial cells. Some bacteria produce hydroxamate-type siderophores, while others produce catecholate-types (Neilands and Nakamura, 1991). In a state of iron limitation, the siderophore-producing microorganisms are also able to bind and transport the iron-siderophore complex by the expression of specific proteins (Nachin *et al.*, 2001; Nudel *et al.*, 2001). The production of siderophores by microorganisms is beneficial to plants, because it can inhibit the growth of plant pathogens (Sharma and Johri, 2003). Ability to selectively chelate iron for own purpose thus making it unavailable for others is a well known mechanism by which antagonistic bacteria limit the growth of pathogenic microbes. Biocontrol by this mechanism is by virtue of siderophore production, and it is well documented in PGPR. There are reports of siderophore production by endophytes too. Hence the five promising isolates were tested for ability to produce siderophores. The result was positive for the three bacterial endophytes *viz.*, EB-31, EB-35 and EB-65. Upon further evaluation under iron dependent production of siderophores, it was found that the bacteria produced more siderophores when there is less amount of available iron in the medium. The isolate EB-65 produced the highest amount, followed by EB-31, and EB-40. EB-35 showed the least value for siderophore production. The differential production of siderophores may be one of the reasons for the differential inhibitory effect exhibited by the isolates against the pathogen. According to Cao *et al.* (2002) endophytic *Streptomyces* which are able to produce siderophore have bio control potential against *Fusarium* wilt disease in banana. Even an endophytic fungus has been reported to produce sideophore under *in vitro* condition (Kajula *et al.*, 2010) whereas, Forchetti *et al.* (2007) reported that none of the bacterial endophytes from sunflower produced siderophores. Though no report of siderophores production by bacterial endophytes are available, Bhavani, (2004) has reported siderophore production by epiphytic bacteria isolated from cocoa.

5.7 INDUCTION OF SYSTEMIC RESISTANCE AND SUPPRESSION OF SEEDLING BLIGHT OF COCOA

The systemic resistance developed in plants by way of beneficial microorganisms is known as ISR or Induced Systemic Resistance (Baker *et al.*, 1997).

The ISR is activated at sites distant from the point of pathogen attack (Dean and Kuc, 1985). The endophyte-mediated resistance was found to be effective over time, indicating persistence, and is hypothesized to be a form of induced resistance (Ganley *et al.*, 2008). Moreover, ISR is suggested as the major mechanism involved in the bio control by endophytes (Adhikari *et al.*, 2001; Bacon and Hinton, 2002; Bargabus *et al.*, 2002; Mishra *et al.*, 2006; Bakker *et al.*, 2007). The five promising endophytic isolates *viz.*, EB-31, EB-35, EB-40, EB-65 and EF-81 were selected based on *in vitro* evaluation of antagonistic activity against *Phytophthora* coupled with growth promoting ability in cocoa seedlings. These were then tested *in vivo* for disease suppression and induction of systemic resistance in cocoa seedlings. For this, another pot culture experiment was laid out to study the efficacy of the isolates in suppressing seedling blight and to assay the defense related compounds produced upon challenge inoculation with *Phytophthora.* During this experiment, the effect on growth parameters of the seedlings was also recorded as ISR also contribute to enhanced growth response and *vice versa.* Certain natural and synthetic compounds stimulate defence responses similar to those observed in resistant host-pathogen interactions. Indirect activators of plant resistance, such as potassium phosphonate, do not act primarily on the pathogen, but reduce the disease by inducing the release of stress metabolites to elicit the defence response (Daniel and Guest, 2006). So it was included as one of the treatments for comparison in addition to Bordeaux mixture which is the commonly used PP chemical against *Phytophthora.* Thus, the promising endophytes were evaluated *in vivo* in comparison with the reference cultures and chemicals (potassium phosphonate and Bordeaux mixture) used for the management of seedling blight of cocoa.

5.7.1 Growth parameters

All the treatments had positive effect on per cent germination of cocoa beans. They had effected early germination also. Accordingly, 100 per cent of the beans treated with EB-31 germinated on the eighth day after sowing compared to less than 50 per cent in the control. EB-35 and Pf_2 also recorded 100 per cent germination by 12^{th} day after sowing. By the final day of observation, EB-31, EB-35, EB-40 and Pf_2 effected cent per

cent germination. Whereas, EB-65, PP, EF-81, Pf_1 and BM registered 98, 96 and 92 per cent respectively while 86 per cent in the control. Other growth parameters *viz.*, seedling height, number of leaves, fresh and dry weight of shoot, girth at collar, length of roots, and fresh and dry weight of roots also were augmented by the treatments. The maximum efficiency (43.5 per cent) in augmenting seedling height was exhibited by isolates EB-31 followed by Pf_1 (32.8), EB-35 and EB-65 (32.6 per cent). The observations on growth parameters confirmed the results obtained in the first nursery experiment since the endophytes selected as promising isolates had pronounced effect on these parameters. However, the additional information obtained here is that, the chemical treatments also effected increase in the growth to a limited extent except that Bordeaux mixture had negative effect on number of leaves at five months after sowing. However, this was not statistically significant compared to other treatments, except EB-31. This result is further reflected on fresh and dry weight of shoots. Hence, it can be assumed that treatment with chemicals also lead to some amount of growth promotion, which may be due to removal of deleterious microbes present in the growing medium and suppression of the disease. As a result of ISR also, growth was enhanced and disease reduced in many crops (Adhikari *et al.*, 2001, Bacon and Hinton, 2002).

5.7.2 Effect on seedling blight of cocoa

The treatments had a profound effect in reducing seedling blight in the nursery after challenge inoculation. The minimum incidence and severity of the disease was recorded on plants treated with EB-31. However, all the isolates including the promising endophytes recorded a lesser disease compared to chemical treatments and control. The results of this nursery experiment indicated that, the isolates could reduce the disease under *in vivo* condition. However, EB-31, EB-35 and Pf_1 were superior to all the other treatments in reducing the per cent disease incidence, while EB-31 and EB-40 were more effective in reducing the disease severity. These isolates were adjudged promising antagonists and growth promoters in experiments conducted earlier in this investigation. Here they have proved their superiority to conventional chemical and biocontrol agents in checking the disease on cocoa seedlings. It is possible that, ISR elicited by these

endophytes might have been operational in the disease reduction exhibited by these organisms. The ability to move and colonise systemically in the plants also might have played a role in their efficiency. Endophytes have been suggested as alternative to systemic pesticides (Rajendran *et al.*, 2006) and the results in the present study supports it. In cocoa reports are many about biological control of various pathogens, like *Phytophthora* sp. (Arnold *et al.*, 2003), *Moneliophthora roreri* (Mejia *et al.*, 2008) by endophytic fungi. However, Melnick *et al.* (2008) have reported the suitability of bacterial endophytes in biological control of cocoa diseases which had been ignored by most of researchers. They have observed induction of broad spectrum resistance and suppression of multiple diseases including black pod rot, frosty pod rot, and witches broom in cocoa, by *Bacillus cereus* isolates.

Of the two chemicals tested, potassium phosphonate was more effective than Bordeaux mixture. Efficacy of potassium phosphonate in the management of *Phytophthora* diseases of cocoa has been reported (Pegg *et al.*, 1985, Anderson *et al.*, 1989; Holderness, 1990). The effect of potassium phosphonate on *Phytophthora* infection was studied in detail by Daniel and Guest (2006) in artificially created *Arabidopsis-phytophthora* pathosystem. They found that, treatment with potassium phosphonate lead to reduction in infection which was confined to a few outer cortical cells in the roots of *Arabidopsis* seedlings and did not progress to the vascular tissue. The host response to phosphonate treatment was accompanied by hyphal disturbances in *P. palmivora*, including swellings outside the plant and hyphal distortions inside the plant tissue.

5.7.3. Assay of defense related compounds and enzymes

ISR is facilitated in plants by the activation of various defense related compounds at sites distant from the point of pathogen attack (Dean and Kuc, 1985). Hence in the present study, major defense related compounds and enzymes involved were assayed at periodical intervals after challenge inoculation. Induction of systemic resistance is an important one among the different mechanisms by which the endophytic

microbes controlled diseases (Cook and Baker, 1983). Moreover, recent reports suggest that, of the wide range of benefits offered by endophytes to plants, inducing plant defense mechanisms (Bargabus *et al.*, 2002; Mishra *et al.*, 2006) is a major one. Hence in this experiment, the compounds involved in ISR due to endophyte treatment were assayed using samples taken from leaves opposite to the challenge inoculated leaves.

5.7.3.1 Total phenol content after challenge inoculation

Several phenolic compounds are present in high concentrations in cells of young fruits, leaves and seeds which have been proposed as responsible for resistance of young tissues to pathogenic mcroorgansms (Agrios, 2005). Some of them occur constitutively while others are induced as part of defense response (Nicholson and Hammerschmidt, 1992). Phenolic compounds enhance mechanical strength of cell wall and also inhibit invading pathogens thereby conferring resistance to the plant, either directly or indirectly through activation of post infection responses in hosts (Harborne, 1988).

In cocoa seedlings treated with endophytic isolates, higher accumulation of phenols compared to control was noticed prior to inoculation with the pathogen. Further, except for Bordeaux mixture all other treatments recorded more phenol content than control before challenge inoculation. There was an increasing trend in phenol content after challenge inoculation, from one DAI to five DAI. The highest content was recorded with EB-35, followed by EB-65, EB-40, EB-31 and Pf_1. It may be noted that the per cent disease incidence was also less with these treatments. Similarly, treatment with the reference cultures and potassium phosphonate also showed more phenol content and less disease compared to control. However, significant increase in phenol content over control was noticed in plants treated with EB-35 and EB-65. Treatments with more phenol content showed less disease indicating the prominent role of phenol in providing resistance against disease. Similar results have been reported in other pathosystems also. Malinowski *et al.* (1998) reported 20 per cent more total phenolic concentration in endophyte infected plants than uninfected plants. Rajendran *et al.* (2006) also reported enhanced mechanical strength of host cell walls and inhibition of invading *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) by endophyte treatment. Benhamou *et al.* (2000)

reported raised levels of phenolics in cucumber roots, affording protection against *Pythium ultimum*. Enhanced accumulation of phenolic materials around the infected cell due to treatment with potassium phosphonate has been reported by Daniel and Guest (2006).

5.7.3.2 Protein content after challenge inoculation

As a result of infection, there is always an increase in protein synthesis especially in the case of incompatible reaction. This is due to enhanced production of defense related enzymes (Agrios, 2005). Synthesis of newer proteins has also been reported (Yamamoto and Tani, 1986) as a result of infection. Such synthesised protein may not be inhibitory by itself but may activate the production of defense related compounds such as phenolics, lignins, phytoalexins *etc.* Protein content in the treated cocoa seedlings was assessed at periodic intervals after challenge inoculation. Before challenge inoculation, the highest content of protein was recorded in Pf_2, however on par with EB-35, EB-40, EB-65 and Pf_1. In general, there was an increasing trend in protein content in all the treatments at one DAI and three DAI which declined thereafter. The endophyte treated plants showed higher protein content compared to control and the highest per cent increase over control was noticed at one DAI in plants treated with EB-35, followed by EB-40, EB-65, Pf_1, Pf_2 and potassium phosphonate. Defence responses triggered by these treatments might have resulted in accumulation of more defence related enzymes and proteins in inoculated plants. There are reports stating N fixation by endophytes which is suggested as a mechanism of growth promotion by them (Stoltzfus *et al.*, 1997; Martinez *et al.*, 2003). Since N is an essential component of protein, it is quite reasonable to expect more protein content in plants with endophytes. The growth promoting effect shown by endophytes also supports this result. Rajendran *et al.* (2006) has reported increase in levels of defense related proteins by pre treatment with PGPE strains, thereby preventing infection with *Xam* in cotton. Here in the present study also, the treatments with higher protein content showed less per cent incidence of seedling blight.

5.7.4 Assay of defense related enzymes

One of the major physiological changes that occur in plants in response to infection by pathogen or stimulation by biocontrol organism is activation of defense related enzymes. Role of phenol oxidizing enzymes in disease resistance is well known. Though these are not directly toxic to the pathogen, enhanced activity of these enzymes is directly correlated with defense reaction in plants. There are enzymes which act directly on pathogens also, aptly called as Pathogenesis Related proteins and β-1,3-glucanase is one among them. Plants develop general resistance in response to infection by a pathogen or other treatment like chemical, stress or beneficial microorganisms. Investigations on mechanisms of biological control by endophytes have suggested induction of plant defence mechanism (Bargabus *et al.*, 2002; Mishra *et al.*, 2006; Bakker *et al.*, 2007) as a major one. Other than phenol, protein, glucosides *etc.*, increase in levels of defense related enzymes has also been reported by various workers as a result of treatment with endophytes. In this study, changes in activity level of PO, PPO and β-1,3 glucanase were assessed using spectral analysis at periodic intervals.

5.7.4.1 Spectronic and electrophoretic assay of peroxidase (PO)

Peroxidase is a phenol oxidizing enzyme which oxidizes phenols to form quinones and also generate hydrogen peroxide. The latter is not only antimicrobial by itself but also releases highly reactive free radicals which in turn leads to polymerization of phenolic compounds to form lignin like substances. These are deposited in cell walls and papillae which interfere with further growth and development of the pathogen. The enzyme peroxidase (PO) has also been implicated in the hypersensitive response, the formation of papillae, and the polymerization of lignin from monomeric lignols (Bestwick *et al.*, 1998; Nicholson and Hammerschmidt, 1992). Peroxidase is the key enzyme in the biosynthesis of lignin (Bruce and West, 1989 and Brisson *et al.*, 1994) and is also reported to be involved in disease resistance and wound healing (Gasper *et al.*, 1982). In general, plants treated with endophytes and the reference cultures recorded higher levels of PO activity. Before inoculation, more than 25 per cent increase in PO

activity over control was expressed by the bacterial isolates *viz.*, EB-31, EB-35, EB-40 and EB-65, Pf_1 and Pf_2. Whereas, EF-81 and the chemicals T_8 (PP) and T_9 (BM) had only 3.9, 1.9 and 0.9 per cent increase respectively. At 1 DAI, also, when the bacterial isolates and PP recorded per cent increase in PO ranging from 26.4 (EB-35) to 60.0 (EB-40), EF-81 had only four per cent increase over control. The activity increased from 1 DAI to 3 DAI and to 5 DAI in all the treatments but the per cent increase over control came down during 3 DAI when the activity was less than control in EB-65 and BM. At 5 DAI the incrase in PO over control was more than 50 per cent in Pf_2, EB-35 and EB-40 and more than 20 per cent in PP, Pf_1 and EB-31. Here also, EF-81 and EB-65 had less increase *viz.*, 12.3 and 5.9 per cent increase respectively. The pattern of PO activity in leaves as described above indicates that, the activity at 1 DAI is crucial in reducing pathogen ingress. In treatments with high PO level at 1 DAI, disease severity was less. Native gel electrophoretic separation of the enzyme extract of leaves expressed six isoforms of PO. More number of isoforms of PO was found in plants treated with endophytes and reference cultures compared those treated with chemicals and control. Induction of more isoforms of PO in ginger due to rhizobacterial treatments has been reported by Vijayaraghavan (2007). Higher levels of PO have been correlated with enhanced ISR in several plants such as rice (Gnanamanickam *et al.*, 1999, sugarcane (Viswanathan and Samiyappan, 2001), chillies (Ramamoorthy *et al.*, 2002). Rajendran *et al.* (2006) had reported cotton plants treated with endophytic bacteria and challenged with *X. axonopodis* pv. *malnacearum* (*Xam*) showed higher levels of PO and less disease. However, the bacterial isolates were more efficient in augmenting PO activity compared to the endophytic fungus. Similar observation had been reported by Uppala (2007) who compared PO activity stimulated by endophytic bacteria and fungi in amaranthus against *Rhizoctonia solani* (Kuhn). Further, according to de Cal *et al.*, (2000) inoculation with *Penicillium oxalicum* prior to infection by *Fusarium oxysporum* f. sp. *lycopersici* reduced the symptoms of the disease but no excess accumulation of defence related compounds other than phenol was associated with it. Hence it may be assumed that, mechanisms such as niche exclusion, antibiosis, growth promotion *etc.*, play major role in the disease reduction effected by endophytic fungi.

5.7.4.2 Spectronic and electrophoretic assay of polyphenol oxidase (PPO)

Polyphenol oxidases (PPO) are responsible for oxidation of phenolic compounds to form quinones which are more toxic to pathogens than original phenols. It is true that, an increased activity of polyphenol oxidases will result in higher concentrations of toxic products of oxidation which in turn lead to greater degrees of resistance to infection. Accordingly, assay of PPO in treated plants which had less disease, showed higher activity of the enzyme at various stages of assessment. Higher activity of PPO over control was noticed in all the treatments before inoculation with a maximum increase of 451.6 per cent over control in T_3 (EB-40) followed by EB-65 and EB-35 and minimum of 116.1 per cent over control in T_1(EB-31). In general, the activity of PPO increased from before inoculation to 3 DAI and declined thereafter to five DAI. There was a sharp increase in PPO activity from before inoculation to 1 DAI and nearly threefold increase at 3 DAI, when, it was the maximum in plants treated with Pf_1 followed by EB-35, EB-31 and Pf_2. Such an enhanced PPO activity immediately after inoculation indicates incompatible reaction in disease development. This increase in PPO is postulated to be due to the change in redox potential of the host (Vidhyasekaran, 1988). This result is supported by less disease in such treatments. As in the case of PO, the bacterial isolates were more efficient in augmenting PPO activity also. Migration of PPO isoforms through native PAGE showed seven isoforms *viz.*, PPO-1 to PPO-7. Five isoforms were present in T_1 (EB-31) and T_4 (EB-65), while T_2 (EB-35) had bands for only four isoforms and T_3 (EB-40) had only two. Treatments T_5(EF-81), T_8 (PP) and T_9 (BM) showed similar banding pattern with three bands each. Whereas, T_6 (Pf_1) had four isoforms and (Pf_2) had all the bands except the band for PPO-7. There are earlier reports of enhanced levels of PPO by endophyte treatments in cotton against *Xam* (Rajendran *et al.*, 2006), in black pepper against *Phytophthora capsici* (Barka *et al.*, 2002), with obvious reduction in disease. According to Chen, *et al.* (2000), PGPE and *Pythium aphanidermatum* elicited PPO activity in cucumber roots. Karthikeyan *et al.* (2005). Cluster analysis of the dendrogam developed based on the isozyme profiles of PO and

PPO showed six clusters. It was found that, treatments T_1 (EB-31, T_6 (Pf_1) and T_2 (EB-35) are distinct from others as they formed separate clusters.

5.7.4.3 Spectronic assay of *β*-1,3-glucanase

ISR leads to broad spectrum resistance to diseases. However, the enzyme β-1,3-glucanase has a prime role in resistance towards oomycetes, as these organisms has glucans as major cell wall component. These are classified under PR-2 group of pathogenesis related proteins and are reported to be associated with greater resistance in plants especially against oomycetes. Healthy plants contain traces of PRPs, but treatment with elicitors such as biocontrol agents, wounding, or stress induce transcription of a battery of genes that code for PRPs (Agrios, 2005). These proteins show strong antimicrobial or antifungal activity. It has also been proved that, β-1,3-glucanases diffuse towards and break down cell walls of pathogenic oomycetes. In the present study, assay of β-1,3-glucanases revealed an increasing trend in all the treatments from before inoculation to one DAI. Thereafter the activity declined till three DAI. In general, the treated plants showed higher activity of β-1,3-glucanase before challenge inoculation, with more than 50 per cent increase over control with Pf_1. At one DAI, the increase was over 75 per cent in Pf_1 and EF-81 followed by EB-31 (66.4 per cent). From 1 DAI to 3 DAI the activity of the enzyme decreased in all the treatments. The results indicated positive relation between high β-1, 3-glucanase activity and disease reduction in cocoa. The present finding is in agreement with reports of other researchers *viz.*, greater levels of β-1, 3-glucanase induced by endophytes against pests and diseases. Maurhofer *et al.* (1994a). Wilhelm *et al.*,(1998) has reported high activity of the enzyme induced by *Bacillus subtilis* an endophyte of chestnut as antagonist against chestnut blight. Jayaraj *et al.*, (2004) has reported the high β-1, 3-glucanase activity in rice induced by foliar application of *B. subtilis* AUBS1 against sheath blight. Radjacommare *et al.* (2005) also had reported enhanced levels of PRPs as a result of treatment with PGPR in ragi.

5.8 Effect of promising endophytes on *Phytophthora* pod rot incidence in the field

The foremost objective of the study was to develop an effective ecofriendly management strategy for *Phytophthora* pod rot of cocoa using endophytes. As Rajendran *et al.* (2006) has opined, the endophytic bacteria may be used as alternative to systemic pesticides in future. Therefore it was essential to test the field efficacy of the five promising endophytes which showed positive effect in growth promotion and disease reduction in cocoa. The performance of the promising isolates was evaluated in comparison with standard cultures of *P. fluorescens* (Pf_1 and Pf_2) and also with the chemicals generally used in the field control of the disease.

The experiment was conducted during June to September 2008 in the existing cocoa garden at the CCRP farm. It was observed that, the efficiency of the treatments varied among the three phases of observation *viz.*, June-July, July-August and August-September. In the beginning, at one week after first spraying, EB-31, EB-40, EB-65 and EF-81 along with Bordeaux mixture recorded more than 90 per cent efficiency over control in checking the incidence of the disease. After three weeks of spraying which coincided with July second week, and when the precipitation was maximum, the endophyte treatments showed higher efficiency compared to the conventional practices. During the second phase also the upper hand of the endophytes especially EB-31, EB-35 was evident, which was on par with Pf_1. Whereas, towards the last phase of the experiment, the efficiency was maximum with Pf_1 followed by EB-35 and Pf_2. The results indicated that, the relative efficiency of the treatments were influenced by inoculum pressure and climatic conditions. When the disease was at its peak during July-August, coinciding with heavy rainfall, the endophytes showed the maximum efficiency. The ability of the endophytes to act as a deterrent in the entry and establishment of the pathogen and their indirect effect in triggering the defence mechanism in the plant might have played a role in reducing the disease incidence. Even though Bordeaux mixture showed efficiency against the disease in the initial phase of the experiment, it was not good during the peak period of disease incidence due to the obvious reasons of wash off there by it becoming less effective. Potassium phosphonate,

an effective chemical against oomycetes, did not show much effect during the initial two phases of the experiment. However, it was effective during the last phase. This may be due to the activation of defence responses in the plant and not due to its direct action on the pathogen. Thus the results of the study suggest the importance of endophytes especially those of bacterial ones in the management of diseases of cocoa. Further, it was noted that, among the endophytic bacterial isolates, EB-31 was the most efficient one as it could maintain the disease incidence at the lowest level during most of the period under observation. It was followed by isolates EB-35 and EB-40 and Pf_1 in reducing pod rot incidence in the field.

A perusal of literature did not show much work on the use of bacterial endophytes in the management of diseases of cocoa especially that of PPR. However, Melnick, (2008) reported the use of endophytic *Bacillus* sp. from tomato against PPR. The efficacy of epiphytic *P. fluorescens* against the disease has been well documented by Bhavani, (2004).

5.9 Characterization of the promising fungal endophyte

Attempts were made to identify the promising fungal isolate EF-81. The isolate produced greyish green conidia which are oval to ellipsoidal, smooth, borne on long, loose or irregular columns. Based on the conidia and conidiophores characters, the fungus was identified as *Penicillium* sp. The isolate was later identified upto species level at the Indian Type Culture Collections (ITCC), Division of Mycology and Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi as *Penicillium minioluteum* (ID No.6905, Ref. No.A 81). Recently, species of *Penicillium* with antagonism towards *Phytophthora* has been isolated from cocoa by Hanada *et al.*, (2010). In India, this species of *Penicillium* has been reported from rhizosphere soil of cassurina from Gujarat (Panda *et al.*, 2010) and also from leaf litter of *Acacia mangium* (Samingan and Sudirman, 2009).

5.10 Biochemical and molecular characterization of promising bacterial endophytes

Attempts to identify the promising isolates were made by studying various cultural, morphological and biochemical characters. Of the four bacterial isolates, three *viz.*, EB-31, EB-40 and EB-65 were Gram negative and one, EB-35 was Gram positive. Based on the cultural, morphological and biochemical characters studied and as described by Harrigan and Mc Cane (1966) and Bergy's Manual of Systematic Bacteriology, Vol. I (Stanley *et al.*, 1989) the bacterial isolates were tentatively identified as *Pseudomonas putida* (EB-31), *Bacillus* sp. (EB-35), *Pseudomonas* sp. (EB-40) and *P. aeruginosa* (EB-65). These isolates were further subjected to molecular characterization by comparison of 16SrDNA sequences revealed that, four promising isolates of endophytic bacteria EB-31, EB-35, EB-40 and EB-65 from cocoa are closely related to *Pseudomonas putida*, *Bacillus subtilis*, *Pseudomonas plecoglossicida* and *Pseudomonas aeruginosa* repectively.

All these four species of bacteria and/or their close relatives were already reported as endophytes from various crops, more over many of them were proved to be efficient biocontrol agents. Many species of endophytic *Bacilli* were isolated from sweet corn, cotton and red clover and chest nut (Mc Inroy and Kloepper, 1995; Sturz *et al.*,1997. Wilhelm *et al.*,1998). Biocontrol efficiency of *Pseudomonas putida* has been reported as early as in 1984 by Colyer and Mount. Moore *et al.*, (2006) characterized endophytic bacterial srtains belonging to 21 genera by comparative sequence analysis of partial 16S rRNA genes and BOX-PCR genomic DNA fingerprinting. Among them there were several species of *Pseudomonas* including *P. putida* and *P. plecoglossicida* isolated from poplar trees. Utilizing 16SrDNA sequence analysis, endophytic strains of *Pseudomonas fluorescens, Bacillus pumilus, P. fulva, B. flexus* and many other bacteria and fungi were identified at the species level by Shi *et al.*, (2009).

Endophytic existence of *P. aeruginosa* is also in accordance with earlier report, where, *P. aeruginosa* in tomato has been reported by Siddiqui and Ehteshamul-Haque (2001). They have also noticed that, root infection caused by the soilborne root-infecting

fungi *Fusarium oxysporum*, *F. solani* and *Rhizoctonia solani* was effectively suppressed following application of *P. aeruginosa*.

5.11 Entry and establishment of endophytes in the plant system

Use of endophytes in bio-control requires introduction of endophytes into plant tissues in quantity, site and life-history stages that effectively antagonize pathogens. However, complete description of inoculation of methods of endophytes associated with trees is scarce (Mejia *et al.*, 2008). Hence in the present study, an experiment was conducted to find out the entry and establishment of endophytic bacteria within cocoa seedlings using radiolabelling. Endophytes have a natural and intimate association with plants, as the internal tissues of plants provide a relatively uniform and protected environment compared to rhizosphere and rhizoplane (Chen *et al.*, 1995). It has been postulated that endophytes originate from rhizosphere or phylloplane and some may be transmitted through seed (Ryan *et al.*, 2008). According to Pleban *et al.* (1995) these bacteria move upward and downward from the point of application, and colonize internal tissues. They find their way to interior of the plant through natural openings.

For this experiment, the promising bacterial isolates were chosen since bacteria are easily multiplied and it is easy to label them. This study was done at the Radiotracer Laboratory attached to College of Horticulture, Vellanikkara. The promising endophytic bacteria were labelled with ^{32}P and used for the study. The results indicated that the promising endophytic bacteria *viz.*, EB-35 (*Bacillus subtilis*) and EB-65 (*Pseudomonas aeruginosa*) are capable of entering the host tissue through intact surface of leaves and pods and move through the conducting tissues of the plant. Entry of bacteria through stomata is a well known fact and this study, suggests the capacity of these novel bio-control agents to establish at the site of infection of the pathogen thereby offering effective protection. However, application on stem and root feeding did not give positive result. Entry of bacteria through root hairs and lateral roots is well documented. But the reason for negative result of the present study may be that, the labeled bacterium failed to reach the shoot portion of the plant within the period of exposure (48h) given, owing

to the use of grown up seedlings for the study or that the isolate was not able to tolerate the radioactivity to which it was exposed which caused attenuation of its systemic movement. With regard to application on the basal portion of the stem also the same may be the reason why the bacteria could not be detected in the upper part of the shoot. Previously, radio labeling has been used successfully to detect the entry and movement of endophytes by Pleban *et al.* (1995). The technique has been used for studying the mode entry and spread of *Ralstonia solanacearum* in tomato seedlings by (Markose, 1996). Autofluorescent protein (AFP) methods are also being utilized to detect and enumerate endophytic microorganisms and to study the courts of entry to plants (Gage *et al.*, 1996; Tombolini *et al.*, 1997).

In conclusion, it is evident that, the five promising endophytic isolates have proved to play a role in growth promotion in cocoa seedling as well as systemic resistance against *Phytophthora palmivora.* Among the endophytes used, isolate EB-31 and EB-35 identified as *P.putida* and *Bacillus subtilis* respectively were effective in reducing the incidence of *Phytophthora palmivora.* The effectiveness was more pronounced during the conditions ideal for large scale flare up of the disease. Other promising isolates also exerted beneficial effect. The enhanced growth promotion and better disease management potentialities of these isolates may be due to the production of plant growth promoting substances and pathogen inhibitory metabolites. Further, enhanced synthesis and activity of defence related compounds and enzymes also might have played in inducing systemic resistance in cocoa against the pathogen. However, more studies are needed on the diversity of antagonistic endophtytes, their role in growth promotion and reducing the incidence of various diseases of cocoa.

Summary

6. SUMMARY

One of the major constraints faced by cocoa farmers of Kerala is the prevalence of fungal diseases, among them, *Phytophthora* pod rot caused by the oomycete *Phytophthora palmivora* is the most serious one. The management practices in vogue include use of fungicides which will be effective only when integrated with suitable cultural practices. Moreover, continuous use of chemical fungicides results in deleterious effects in the ecosystem. Here comes the significance of biocontrol agents, which are ecofriendly. However, the success of conventional biocontrol agents is limited in those diseases which affect the aerial plant parts. It is here, the research on antagonistic endophytes becomes valid, as they possess the beneficial attributes of other biocontrol agents like the capacity to induce systemic resistance and growth promoting ability with the additional advantage of systemic existence in the plants. Hence, the present investigation was carried out with the objective of harnessing the potential of native endophytic microbes of cocoa for the management of PPR. The salient findings of the study are summarized below:

1. The pathogen causing PPR of cocoa was isolated and its pathogenicity established. The cultural and morphological characters of the pathogen confirmed it as *Phytophthora palmivora* (Butler) Butler.

2. Enumeration of endophytic microorganisms from different parts of cocoa plants grown at various locations of the major cocoa growing areas of the state revealed the predominance of bacteria and fluorescent pseudomonads compared to filamentous fungi and yeasts.

3. The endophytic population was more in roots compared to other parts of the plant. Altogether, 325 endophytic isolates were collected including 116 bacteria, 153 fluorescent pseudomonads, 34 yeasts and 22 fungi.

4. Of the 325 endophytic isolates subjected to preliminary screening, 82 were found to be antagonistic to the pathogen.

5. Among the antagonistic endophytes also, there were more fluorescent pseudomonads (29) and bacteria (28), compared to filamentous fungi (four) and yeasts (21).

6. Out of the 82 antagonists, 44 exerted more than 40 per cent *in vitro* inhibition of the pathogen, and these included 18 bacteria, 22 fluorescent pseudomonads and four fungi. Of these 44 isolates, 25 were able to bring about more than 55 per cent reduction in pod rot infection on detached cocoa pods. These, when tested on detached leaves, it was found that, all the 25 isolates effected more than 70 per cent reduction in the infection on leaves. These were selected for the nursery experiment for testing their growth promoting effect. These 25 isolates included 9 bacteria, 12 fluorescent pseudomonads and four fungi.

7. In general, endophytic isolates expressed a positive effect on the germination of cocoa beans, seedlings height and number of leaves. Eleven isolates enhanced fresh weight of shoot while 18 had positive effect on dry weight. With regard to the root parameters, 20 isolates augmented root length and root fresh weight while 18 had positive effect on dry weight of root. The growth promoting effect was more pronounced in sterilized potting mixture.

8. Of the 25 isolates evaluated for growth promoting efficiency on cocoa seedlings, eight *viz.* EB-19, EB-22, EB-31 EB-35, EB-40, EB-65 and EF-81 showed profound effect on various growth parameters studied and they had good antagonistic ability also. These were selected as potential endophytes for further studies and these included isolates of two bacteria, five fluorescent pseudomonads and one fungus. It was noted that, six out of these eight potential isolates were obtained from pods and two from leaves.

9. The eight potential endophytic isolates were tested in *in vitro* for various attributes that lead to their antagonistic and growth promoting effect. Two reference cultures *viz.*, Pf_1 (*P. fluorescens* from KAU) and Pf_2 (*P. fluorescens* from TNAU) were also included for comparison. The isolates EB-35, EB-40, EF-81 and Pf_1 produced more ammonia and EB-19 the least. P solubilizing ability was maximum for EB-35 followed by EB-22 and

Pf_2. Three isolates *viz.*, EB-35, EB-40 and EB-65 produced high quantity of IAA. However none of the isolates produced HCN. The antagonism index (AI) was the highest for EB-31. The highest value for vigour index (72.3) was recorded for seedlings treated with EB-31.

10. Based on the aforesaid parameters, Plant Growth Promoting Index (PGPI) of the eight potential endophytes and two reference cultures was worked out, and it was found that, out of the eight endophytic isolates five *viz.*, EB-31, EB-35, EB-40 EB-65 EF-81 and the reference culture Pf_1 had PGPI above 60.

11. The five endophytic isolates which were assigned maximum score for PGPI were selected as promising endophytes. There were subjected to further detailed investigation on the mechanism of action and induction of systemic resistance.

12. Antibiosis test for volatile inhibitory metabolites revealed that, all the isolates tested *viz.*, five promising endophytes, Pf_1 and Pf_2 produced volatiles which inhibited the growth of the pathogen.

13. Production of non-volatile, diffusible inhibitory substances was also tested and the result was positive for all the isolates. Maximum inhibition of the pathogen by way of non–volatile inhibitory metabolites was effected by EB-40 and EB-35.

14. Isolates EB-31, EB-40, EB-65 Pf_1 and Pf_2 emitted fluorescence under UV light. Test for iron dependant production of siderophores revealed negative relation between concentration of Fe^{3+} ions in the medium and sidophores production by a bacterial isolate.

15. The five promising endophytic isolates were tested *in planta* for induction of systemic resistance in cocoa, in comparison with reference cultures and chemicals including Bordeaux mixture and potassium phosphonate.

16. Seed treatment, followed by foliar and soil application of endophytic isolates augmented the growth of cocoa seedlings. Here also the endophytes had more profound effect on growth parameters compared to the chemicals. Incidence and severity of seedling blight was also reduced significantly by endophytes with minimum incidence and severity of the disease recorded with EB-31.

17. In general, growth parameters were augmented by all the treatments with maximum seedling height recorded by EB-31 and Pf_1.

18. The chemicals also had a effect on growth parameters except Bordeaux mixture which showed a negative effect on number of leaves which was reflected in fresh and dry weight of shoots.

19. Defence related compounds and enzymes in the leaves were assayed before and after challenge inoculation. Increasing trend in total phenol content was observed in treated plants from before and to five days after inoculation with maximum increase in EB-35. Protein content increased from before to three DAI and the maximum increase was in EB-35.

20. In general, there was an increase in protein content at one day and three DAI. Protein content estimated spectroscopically revealed significant increase due to various treatments including endophytes with maximum increase in EB-35.

21. Profound increase in peroxidase activity was observed in treated plants at before inoculation and also at 1, 3 and 5 days after inoculation. Maximum increase was noticed in plants treated with EB-40. Appreciable increase in polyphenol oxidase was noticed in treated plants with maximum increase in Pf_1. Native PAGE analysis revealed six isoforms of PO and seven isoforms of PPO. More isoformes were present in plants treated with endophytes. Cluster analysis based on PO and PPO isoforms revealed six clusters with EB-31, EB-35, Pf_1.

22. Spectronic assay of β-1, 3 glucanase revealed enhanced production of the enzyme in response to endophyte treatment followed by challenge inoculation with the pathogen. Maximum increase was in plants treated with Pf_1, followed by EF-81.

23. Field experiment revealed efficacy of promising endophytes in the management of PPR. Efficacy of the treatments varied among the three phases of the field trial. In the first phase *viz.*, after the first spraying, delay in incidence of the disease was noticed in EB-31 and EB-35. At the end of the first phase, minimum disease incidence was recorded in EB-65, EF-81 and BM.

24. During the second phase, the disease showed an increasing trend in all the treatments, however after three weeks of second spraying, the least incidence was in EB-31 and the maximum in control.

25. In the third phase, the disease declined in all the treatments with recession in monsoon. After two weeks of third spraying, the minimum disease was in Pf_1, followed by EB-35 and maximum in control.

26. In general, the treatment with endophytic isolate EB-31 had a positive effect in reducing the PPR of cocoa especially when the disease was at its peak.

27. Based on the cultural, morphological and biochemical characters, coupled with results obtained in the 16 Sr. DNA sequencing data , the promising bacterial isolates EB-31, EB-35, EB-40 and EB-65 were identified as *Pseudomonas putida* , *Bacillus subtilis P. plecoglossicida, P. aeruginosa* respectively. The promising endophytic fungus EF-81 was identified as *pencillium minioluteum*.

28. Radiotracer studies revealed entry and colonization of endophytic bacteria EB-35 and EB-65 in the conductive tissues on application on intact surface of cocoa leaves and pods.

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Appendix

APPENDIX–I

1. Carrot agar (CA)

Carrot	:	200g
Agar	:	20 g
Distilled water	:	1000 ml

2. Potato dextrose agar (PDA)

Potato	:	200g
Dextrose	:	20g
Agar	:	20g

3. Nutrient agar medium (NA) (pH 7.2)

Peptone	:	20 g
Beef extract	:	1.0 g
NaCl	:	5.0 g
Agar	:	20.0 g
Distilled water	:	1000 ml

4. Martin's rose bengal streptomycin agar medium (MRBSA)

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

5. **Kenknights agar (pH 7.0)**

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

6. **Glucose yeast extract agar peptone (GYEPA)**

Glucose	:	20g
Yeast extract	:	5g
Peptone	:	5g
Agar	:	20g
Distilled water	:	1000ml

7. **Kings'B media (KB) (pH7.2)**

Peptone	:	20 g
Glycerol	:	10 ml
K_2HPO_4	:	1.5 g
$MgSO_4\ 7H_2O$	:	1.5 g
Agar	:	20.0 g
Distilled water	:	1000 ml

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

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

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

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

10. **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	:	1000ml

11. **Glucose mineral medium (pH 7.2)**

Glucose	:	30.0 g
NH_4SO_4	:	2.0 g
KH_2PO_4	:	3.0 g
$Mg\ SO_4$	:	0.5 g
Distilled water	:	1000ml

12. **Nutrient broth glucose media (NBG) (pH 7.2)**

Peptone	:	5.0 g
Beef extract	:	1.0 g
Yeasty extract	:	2.0 g
NaCl	:	5.0 g
Glucose	:	20 .0 g
Distilled water	:	1000ml

APPENDIX-II

I. Reagents for isozyme analysis

a. 30 % Monomer solution

30% Acrylamide	: 60.0g
60% Bisacrylamide	: 1.0g

b. 4X Resolving buffer

1.5M Tris base	: 36.3g
Distilled water	: 200ml
pH	: 8.8

c. 4X Stacking gel buffer

0.5M Tris base	: 3.0g
Distilled water	: 50ml
pH	: 6.8

d. Ammonium per sulphate : 10 %

A. Preparation of separating / resolving gel mix (8 per cent)

30% monomer solution	: 2.7 ml
4X resolving buffer	: 2.5 ml
Distilled water	: 4.69 ml
Ammonium persulphate	: 100 µl
TEMED	: 10 µl

B. Preparation of stacking gel mix (6 per cent)

30% monomer solution	: 0.67 ml
4X stacking gel buffer	: 1.25 ml
Distilled water	: 3 ml
Ammonium persulphate	: 25 µl
TEMED	: 10 µl

II. Buffers used in Native Polyacrylamide Gel Electrophoresis (Native PAGE)

A. Sample Buffer (5X)

Tris HCl (pH 6.8)	: 5.0ml
Sucrose	: 0.5g
Mercaptoethanol	: 0.25 ml
Bromophenol blue	: 1.0 ml
Distilled water to	: 10.0 ml

Dilute to 1X and use.

B. Electrode buffer

0.05M Tris	: 6.0g
0.192M Glycine	: 14.4g
Distilled water to	: 1000ml

APPENDIX-III

I. Reagents for DNA isolation from bacteria as per Pitcher *et al.* (1989)

A. 0.5 M EDTA pH 8.0

Prepare 1000 ml using

Sodium EDTA : 186.1g

NaOH :20g

Deionized water :800ml

(Adjust pH with 5N NaOH, and adjust final volume with deionized water)

B. 100X 1.0 M Tris, 0.1 EDTA

Tris HCl : 121g

Distilled water : 600ml

Adjust pH 8.0 by adding 42ml conc.HCl

0. 5 M EDTA pH 8 : 200 ml

Adjust to 1000ml with deionized water

C TE Buffer

(Tris HCl -10.0 mM; EDTA -1.0 mM)

Prepare 1000ml using

100 X TE Tris, pH 8.0 : 10 ml

Distilled water : 990 ml

D Guanidine thiocyanate–EDTA-Sarkosyl (GES)

Prepare 100ml GES solutionusing

Guanidine thiocyanate : 60g

0.5 M EDTA pH 8.0 : 20ml

Add 20 ml deionized water

Dissolve all ingreidients at 65°C, cool to room temperatue

Add 1.0g N – Lauryl sarkosyne

Adjust volume to 100ml with distilled water, filter sterilize using 0.45µm filter, store at room temperature. (Note: Guanidine thiocyanate is harmful, use suitable protective wear)

E Resuspension buffer 0.15 M NaCl, 0.01M EDTA, pH 8.0

Prepare 1000ml using

NaCl : 8.77g

0.5M EDTA pH 8.0 : 20ml

Adjust to 1000ml using distilled water

II. Buffer and dye used in Agarose Gel Electrophoresis

A. 6X Loading / Tracking dye

Bromophenol blue : 0.25 %

Xylene cyanol : 0.25 %

Glycerol : 30 %

The dye was prepared and kept in refrigerator at 4°C

B. 50X TAE buffer (pH 8.0)

Tris base : 242.0 g

Glacial acetic acid : 57.1 ml

0.5 M EDTA (pH 8.0) : 100 ml

Distilled water to : 1000 ml

The solution was prepared and stored at room temperature

APPENDIX-IV

Sequence 7 **(Cult No.35)**

GNGNGNTNNNNCNGTCTNACTGCAGTCGAGCGGATGACGGGAGCTTGCTC
CTTGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAG
TGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGG
AGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGG
ATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTG
GTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCC
AGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAG
TTGGGAGGAAGGGCAGTAAGCTAATACCTTGCTGTTTTGACGTTACCGAC
AGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGG
TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCTCGTGTAGGTGCTCGTAAATG
AAGCANGTGAAAGCANNGGCTCGANGGTCGGCAAGTGCAATCCATAGAGTCGTA
GCTAGAGTGGCGGTAGATGGCTGATGGAATCTACTGNGTACCGGTGAAACGCGT
AGATATTGTAAGGAACTCCAGTGGCGAGTGCGCACACTTGGCCGCATACTGACA
CTGAGTAGCGAAAGCTTGGTGAGCACCCAGGATTAGCTCCCCAGGTAGTGCACC
CCGTCAGGGATTTCAACTAGCCGTTGGAATCCCTGAGATTTTAGTCGCGCAGCTA
CCGCACTCAGTAAATCGCATCGGGATTACGGCCGCAAGATTAAAACTCAAATGA
ATGCAGTGGGCCCCGCACAACCGGGGGAGCATCTTGTTTAATTCGAAGCAACGC
GAAGACCCTTACCAGGCCTTAACATGCAGAGAACTTTCCAGAGATGGATTGGTG
CCTTCGGGAACTCACACACAGGTGCGGCATGGCTGTCGTCAGCTCGTCAAAACA
GATGTTGGGTTAAGTCCCGTAACGAGCTCCAACCCTTGTCCTTAGTTACCTCCAC
GTTCTGGTGGGCCCCCTAAGGAGACTGCGGGTCACAAACTGGAGGAAGGTGGGG
ATGACGTCAAGTCATCATGGCCCTTACGTCCTGGGCTACTCTCATGTTACAATGG
TCGGTTCAGAGGGTCGCCAAGCCGCGAGGTGGAGCTAATCTCACAAAACCGATT
GTAGTCCGGATTGCAGTCTGCAACTCGCATGCGAGAAGTCGGAATCGGTAGTAA
TCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTTAACACACCGCCCG
TCACACCATGGGACTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGGA
CGGTTCCCACGGTGTGATTCATCACTAGGGGAAGTCNNNGTCGNNGANNNGNNA
A

Sequence 6 (**Cult No.31**)

NNGNAAGGTGGNNGCTCGTANNACTGCAGTCGAGCGAACGGATTGGACTT
GGTCCCTGATGTTCCCGGCGGAGGGGTGTGTAAAACGTGGGTAACCTCTC
TGTAAGACTGGGGTAACTGCCGGAAACCGGGGGGAATACCGAATGGGTGT
CTGAACCCCGGGCCCGGCACATGTGAAGTGGCTTCGGGGACCACTTATAG
ATAGACCCCCGGCTCATTATCTATTTGGGGTGGTAACACCTCACGAGGCG
ACGATACGTGGCCCACATGAGAGGGTGATCGGCCACACTGGGACTCAGAC
ACACCGCACACGCTTATAGGAGGCACAGGCAAGGAATCTTCCCCAATGGA
CAAAAGTCTGACACAGCAACGCCCCGCGAGTGATAAAGGTTTTTTGATGT
TGTAGCTCTGTTGTTTGGGAAAAAAAAATGCGTTTCAAAAAGGGGGGCGC
CTTGACAGTACCTAAACCCAAAACCCTGCCTAACAACATGACCCCACCCC
CGGTAAAATATATGAGGCAAGCGATGTCTGGAAATATTTTTCGCGTAGAG
CTCACACACGTTATCTTATGTCTGAGATGTGAGCCCCCGCCTCAACTGCG
TAGAGTCTCTGTGAACTGTCGGACTTGTGTGCGCAGAATGAGAGTGGAGT
TCTACGCGTAGCGGTGAATGCGCACACACGTGTAGTAACACCTCTGTGGA
GAGCGACTCGTAAACGAAGGGTTTTTATTTTAGGGTGTTCCCCCCCCTTGGGCCG
CAGATAACGCAATAGGCATTCCCCTGGTGAGATCGGCCGAAAGCCTGAAACCCA
AAGCAATGGGCGGGGGCCCCCCCAACGCGGGGAGCATTGGTTTATTTGAAAGGA
ACGGGAAGAACCTTTCCGGTTCTTGATTTCCTCCGACATTCCAAGAGATAGGGTC
TCCCCTTGGGGGCAGAGTGACCGGGGTTCATGGTTGTCGTCAGCTCGTTTCTTGA
GATGTTTTTTTAATTCCCGCAACAAACGGACCCCCTTGTTCATAGTTCCCAACATT
CAGTTGGGCATTCTAAGGTGAGGGCCGGTGTCAACGGGGGGTAGGGGGGGAGG
ACCTCAAATCATCATGCCCCTTATGACTCGGGCCCCACACGTGCTCCCATCGTCT
CAACAAAGACAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCCTTTCTCAT
TTCGGATGGCAGTCTCCAACTGGCTTGCGTGAAGCCGGATTCCCTTTTAATCGCG
GATCAGCATGCCGCGGTGAATAGGTTCCCCGCCCTTGTACACACCTCCCCCCCCA
CCACGAGAGTTTTAACACCTGAAATGGCTGAGGTAACCTTTTTGGACCCAGCCGC
CAAAGGTGGGACAGATGATGGGGGATNNNGNNNNNNAAGNNGGTCCCNNCNCC
C

Sequence 8 (**Cult No.40**)

GNNNNTTNNGGNNNNNNNTNCTGCAGTCGAGCGGACAGATGGGAGCTTGCT
CCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGT
AAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGTTTG
AACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATG
GACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAAC
GATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACG
AAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTA
AAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCT
TGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCT
CGCAGGCGGTTTCTTAAGTCTGATGTGAAATGTCNGNATTANNNCGTANGTNTGA
CTGACGCTCNNNNANCNNGNTGTGAACGCCCCCNNNTCATCCGTGTANGGTCAT
GTCATGCTGGCGNCTGACTGCAGAGATGAGTANTGCAGTNCACGTGTAGCTGAT
GAAGTGCGTAGAGATGTAGCAGTACACCAGTGCGAAGCCGACTCTCTGGTCTGT
ATCTGACGTCTGAGTAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGG
TAGTACACTCGGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGT
GCTGCAGCGAACGCATTAAGCACTCCGCGTGGGGAGTACGGTCGCAAGACAGAA
ACTCTAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTG
GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGA
TAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTT
GCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTA
CAATGGACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAA
TCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGC
TAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACAC
CGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTA
GGAGCCAGCCGCCGAAGGTGGGACAGATGATGGGGTGAAGTCGANNNTNNNAG
NN

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Abstract

ENDOPHYTIC MICROORGANISM MEDIATED SYSTEM RESISTANCE IN COCOA AGAINST *Phytophthora palmivora* (Butler) Butler

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ABSTRACT OF A THESIS

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ABSTRACT

The study on 'Endophytic microorganism mediated systemic resistance in cocoa against *Phytophthora palmivora* (Butler) Butler' was carried out during 2005-2010. The pathogen causing pod rot of cocoa was isolated from infected pods, and its pathogenicity established. Based on cultural and morphological characters, it was identified as *Phytophthora palmivora* (Butler) Butler. Endophytes were isolated from samples of feeder roots, tender shoots, leaves and pods of cocoa collected from various locations of major cocoa growing areas of the state. The population of endophytic microflora varied among different locations and parts of the plant, and in general, the population was more in roots. Bacteria and fluorescent pseudomonads were more abundant than filamentous fungi and yeasts.

Out of the 325 endophytic isolates comprising of 116 bacteria, 153 fluorescent pseudomonads, 34 yeasts and 22 fungi, 82 were found exerting antagonism towards the pathogen. These antagonistic endophytes were further evaluated in *in vitro* by dual culture and by inoculation on detached cocoa pods, and leaves. It was found that, 25 *isolates* were more efficient antagonists. These included endophytic isolates of 12 fluorescent pseudomonads, nine bacteria and four fungi. The 25 efficient endophytic antagonists were evaluated for growth promoting ability in cocoa seedlings. It was observed that eight isolates had a profound effect on growth promotion. Hence, these were selected as potential endophytes and were subjected to various tests to study the attributes underlying their antagonistic and growth promoting effects. The potential endophytes consisted of isolates of two bacteria, five fluorescent pseudomonads and one fungus. These eight potential endophytes along with two reference cultures were evaluated in *in vitro* for various attributes, which underlay their beneficial effects. It was found that, isolates EB-35, EB-40, and EF-81 produced more ammonia. Phosphate soulubilizing ability was maximum for EB-35. The isolates EB-35, EB-40 and EB-65 produced high quantity of IAA. High score for antagonistic index was obtained by EB-31 and EB-35. Vigour index of coca seedlings was also high for EB-31 and EB-35. The plant growth promoting index worked out based on aforementioned attributes was high for five isolates *viz.*, EB-31, EB-35, EB-40, EB-65 and EF-81, which were selected as

promising endophytes and were subjected to further studies and *in vivo* evaluation. The promising endophytes were found to produce volatile and non-volatile inhibitory metabolites against the pathogen. Maximum inhibition through volatile production was with EB-35 and EB-31. While through non-volatiles, the maximum inhibition was by EB-35 and EB-40. Of the four promising bacterial endophytes, three emitted fluorescence under UV light and EB-31 and EB-65 produced more siderophores under iron limiting condition.

A pot culture experiment was conducted to study the induction of systemic resistance and suppression of *Phytophthora* infection in cocoa seedlings by the promising endophytes. Here also, endophytic isolates effected growth promotion in cocoa seedlings and reduction in infection. Induction of systemic resistance was studied by assay of defense related compounds and enzymes. In general, the study revealed more accumulation of phenols and proteins in treated seedlings. Higher activity of PO, PPO, and β-1,3-glucanase was also noticed. Native PAGE analysis revealed six isoforms of PO and seven of PPO. More isoforms were present in endophyte treated plants. Promising endophytes were evaluated for efficiency in reducing *Phytophthora* pod rot in field in comparison with two reference cultures and chemicals. During the first phase of experiment, *viz.*, after first spraying, maximum reduction in disease was observed in EB-65 and EF-81. Whereas, after second spraying least incidence was in EB-31. After two weeks of third spraying, minimum disease was observed in Pf_1 and EB-35. However, the isolate EB-31 was the most efficient one which recorded the least disease incidence during most of the period under observation especially when the disease was at its peak.

Based on cultural, morphological and biochemical characters coupled with results of molecular characterization, the promising bacterial endophytes were identified as *Pseudomonas putida* (EB-31), *Bacillus subtilis* (EB-35), *P. plecoglossicida* (EB-40) and *P. aeruginosa* (EB-65). The isolate EF-81 was identified as *Penicillium minioluteum*. In the radiotracer experiment, it was found that EB-35 and EB-65 entered the cocoa seedlings when applied on leaves and also inside the pods on application on the intact surface.