ECO-FRIENDLY APPROACHES FOR THE MANAGEMENT OF GROUNDNUT LEAF BLIGHT CAUSED BY *Alternaria alternata* (Fr.) Keissler

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Thesis submitted in part fulfillment of the requirements for the degree of ***DOCTOR OF PHILOSOPHY*** *(AGRICULTURE) in* ***PLANT PATHOLOGY*** *to the Tamil Nadu Agricultural University, Coimbatore - 641 003*

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CERTIFICATE

This is to certify that the thesis entitled "**ECO-FRIENDLY APPROACHES FOR THE MANAGEMENT OF GROUNDNUT LEAF BLIGHT CAUSED BY *Alternaria alternata* (Fr.) Keissler**" submitted in part fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY (AGRICULTURE) IN PLANT THOLOGY** to the Tamil Nadu Agricultural University, Coimbatore, is a record of bonafide research work carried out by **Miss. K.CHITRA** under my supervision and guidance and that no part of this thesis has been submitted for the award of any other degree, diploma, fellowship or other similar titles or prizes and that the work has not been published in part or full in any scientific or popular journal or magazine.

Place: Coimbatore-3 **(Dr. N. RAGUPATHI)**
Date: Chairman

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(Dr. P. NAGARAJAN)

Date: **External Examiner**

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ABSTRACT

ECO-FRIENDLY APPROACHES FOR THE MANAGEMENT OF GROUNDNUT LEAF BLIGHT CAUSED BY *Alternaria alternata* (Fr.) Keissler

By

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DEGREE : Doctor of Philosophy
in **Plant Pathology**

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An intensive survey conducted in ten major groundnut growing districts of Tamil Nadu, during Rabi 2003, revealed that, Alternaria leaf blight incidence was observed almost all the districts with per cent disease index ranged from 33.77 to 63.12. Among the bioagents tested five *Pseudomonas fluorescens* strains and ten *Bacillus subtilis*(Cohn) strains tested under *in vitro* conditions for the inhibition of radial mycelial growth of the pathogen *Alternaria alternata* ((Fr.) Keissler), the strains Pf1 and BS1 recorded maximum inhibition. Among the sixteen plant species tested, the leaf extract of *Abutilon indicum* and *Datura metel* recorded maximum inhibition of the radial mycelial growth of the pathogen.

Seed treatment with *P. fluorescens* Pf1 significantly increased the germination, shoot and root length of groundnut. Maximum production of indole acetic acid, salicylic acid, siderophores and hydrogen cyanide content was recorded by this strain. The

phenolic and alkaloid compounds isolated from *A. indicum* and *D. metel* inhibited the radial mycelial growth of *A. alternata*.

Maximum activity of phenyl ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), tyrosine ammonialyase, chitinase and β-1,3 glucanase activity and enhanced accumulation of phenol and protein content were observed in groundnut plants treated with *P. fluorescens* Pf1 strain and challengeinoculated with *A. alternata*. Expression of PO2, PPO1 and PPO2 isoforms was observed in all the plants treated with biocontrol agents, while additional PO1, PPO3, PPO4 and PPO5 was observed in biocontrol agents treated plants, followed by challenge inoculation of the pathogen. Studies on the expression of proteins through SDS PAGE revealed that, pre-treatment of groundnut plants with biocontrol agents induced 14 kDa and 25 kDa proteins. Plants treated with biocontrol agents followed by challenge inoculation with the pathogen induced additional proteins with 35, 40, 55 and 97 kDa proteins. Healthy and inoculated control plants showed ~14 and 25 kDa protein induction.

In pot culture condition, seed treatment and foliar spraying of biocontrol agents *viz*., Pf1, Pf2, BS1 and BS5 and foliar spraying of leaf extracts of *A. indicum* and *D. metel* were highly effective in reducing the disease incidence. The results of three field trials revealed that, seed treatment with Pf1 @ 10 g/kg of seed, followed by two foliar sprays (0.2%) on 30 and 40 days after sowing significantly reduced the disease incidence and increased the yield.

By using indirect ELISA, *A. alternata* could be detected as early as four days after inoculation. In Rabi 2002-03, (first sowing) there was a positive correlation between maximum temperature, minimum temperature and growing degree days and disease development, while in the second sowing minimum temperature, GDD and evening relative humidity were significantly associated with the disease development. In kharif

2003-04 there was a positive correlation of diurnal variation (DV) for first sowing and morning RH for the second sowing. Interestingly, in the third sowing, all the weather parameters had no influence on the disease. In Rabi 2003-04, minimum temperature and GDD had positive correlation with the disease in all the three sowings. Maximum temperature and bright sunshine hours had positive correlation with the disease for first sowing and maximum temperature and evening relative humidity had contributed for the disease development, for second and third sowing respectively.

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CHAPTER I

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is one of the most important oilseed crops cultivated extensively throughout the tropic and warm temperate regions of the world. It plays an important role in the economy of several countries. In India, it is cultivated in an area of 67.32 lakh ha with a total production of 62.22 lakh metric tonnes with an average yield of 924 kg ha^{-1}. In Tamil Nadu, it is cultivated in an area of 8.25 lakh ha with a total production of 14.57 lakh metric tonnes with an average yield of 1,765 kg ha^{-1} (Singhal, 2003).

Groundnut production is limited by several factors and among these, diseases play a major role. The crop is affected by nearly 35 fungal and 10 virus diseases. Among the fungal diseases, leaf blight caused by *Alternaria alternata* (Fries) Keissler is one of the destructive diseases, which causes economic loss upto 40-50 per cent (Mathur and Sarbhoy, 1997).

For the management of groundnut leaf blight, researchers mainly concentrated only on fungicides, which yielded success only to a limited extend, but encountering with undesirable problems. Large-scale utilization of chemical fungicides resulted in deterioration of soil fertility, residual toxicity, development of resistance by the pathogen and loss in biodiversity. To alleviate all these ill effects, environmentally safe, long lasting and eco-friendly, non chemical means are in need for effective plant disease management.

Biological method of plant disease control is an alternative method to chemical fungicides, as it is safe, effective, economical and ecofriendly. In the recent years, there is growing awareness about the use of biocontrol agents such as *Trichoderma* spp. *Bacillus*

subtilis and *Pseudomonas fluorescens* to control fungal diseases in different crop plants (van Loon *et al*., 1998).

The information on the occurrence of the disease, epidemiology and eco-friendly management practices using biocontrol agents and the possibility of enhancing the levels of resistance in susceptible cultivars by inducing resistance has not been exploited till date. In view of the above mentioned research gaps, hence the following investigations were taken.

1. Survey for the occurrence of leaf blight caused by *Alternaria alternata* in major groundnut growing districts of Tamil Nadu.
2. Evaluation of biocontrol agents and plant products against the disease under *in vitro*, pot culture and field conditions.
3. Epidemiology of the disease.
4. Studies on the physiology of groundnut plants treated with biocontrol agents and plant products.
5. Production of antiserum for detection of the disease.

CHAPTER II

REVIEW OF LITERATURE

The leaf blight of groundnut, caused by *Alternaria alternata* (Fr.) Keissler is almost co-existent with the crop and contributes to significant yield loss throughout the world (Mathur and Sarbhoy, 1997). The disease could be controlled effectively by using chemical fungicides. Development of fungicide resistance by the pathogen, groundwater and food stuff pollution and the development of oncogenic resistance have encouraged for the exploitation of non chemical means of disease management. Information available on these lines are reviewed herein. Recently Rhizobacteria, fluorescent pseudomonads and *Bacillus* have received much attention as good biocontrol agents for the management of both soil borne and foliar pathogens through several mechanisms (Wilson *et al*., 1995).

2.1. Occurrence of the disease

A leaf spot disease of groundnut caused by an unknown species of Alternaria was first reported from the Ukraine region of the USSR by Bohovik (1936). Subsequently, it has been reported from Barbados (Norse, 1974) and Uganda (Mukiibi, 1975). In India, groundnut leaf blight disease due to Alternaria was first reported from Punjab (Aulakh, 1969) as a minor disease. Thereafter the occurrence of the disease was recorded in Tamil Nadu (Balasubramanian, 1979) and Karnataka (Subrahmanyam *et al*., 1981).

2.2. Symptoms

Bohovik (1936) described the symptoms of Alternaria as brown spots with a darker margin and light centre, round or irregular, which coalesce frequently. The affected leaves became chlorotic and died prematurely. Aulakh (1969) and Ghewande *et al*. (1982) observed blighting of apical portion of leaflets, which turned light to dark brown. In the later stage of infection, blighted leaves curled inward and became brittle.

The lesions appeared on the top leaves. Kulkarni (1974) noticed brown and irregular lesions surrounded by yellowish halo. Mukiibi (1975) observed light-brown lesions starting from the margin of the leaflets eventually covering half of the leaf surface. The lower surface of the lesion was covered by a light to heavy olive-green sporulation. Subrahmanyam *et al*. (1981) have observed small, chlorotic, water-soaked lesions that spread over the surface of the leaf.

2.3. Pathogen (*Alternaria alternata)*

Aulakh (1969) reported *Alternaria tenuis,* the incitant of leaf blight of groundnut as the synonym of *A. alternata. A. alternata* produced often branched chains of conidia, obpyriform, obclavate, ovoid or ellipsoidal with a short cylindrical or conical beak, sometimes one third length of the conidiophore. Conidia were pale to mid-golden brown in colour, smooth or verrucose with up to eight transverse and usually several longitudinal or oblique septa. The length of the spores ranged from 8.5 to 37.8 µm and the width ranged from 6.3 to 8.4 µm with an average of 24.9 x 7.9 µm (Simmons, 1965).

2.4. Biological control of foliar pathogens

Species of *Pseudomonas* and *Bacillus* are currently considered as the most effective bacteria for biological control of both soil borne and foliar diseases. They are easy to isolate and grow in the laboratory and they are not fastidious in their requirement of nutrition. Since they are normal inhabitants of the soil and especially root surfaces of plants, they grow well on the root surfaces when introduced artificially. They are known to produce a variety of antibiotics and siderophores, some of which are active in soil. They have been implicated in many cases of natural biological control and plant growth promotion (Kloepper *et al*., 1980).

Prasad and Kulshrestha (1999) reported that *Pseudomonas* and *Bacillus* spp. as seed treatment, were effective in inhibiting *A. helianthi* under *in vitro* condition. Hebbar *et al.* (1991) found that the bacteria associated with leaves and roots inhibited *in vitro* growth of *A. helianthi.* Sukumar and Ramalingam (1986) reported that application of *P. maltophila* to mulberry leaves led to reduction in leaf spot incidence caused by *Cercospora moricola. Pseudomonas cepacia* formulated as wettable powder effectively controlled peanut leaf spot under field conditions (Knudsen and Spurr, 1987). Savithri and Gnanamanickam (1987) reported that seed bacterization of peanut with *P. fluorescens* reduced the groundnut root rot caused by (*Rhizoctonia solani*) under greenhouse condition.

Fluorescent pseudomonads have revolutionized the field of biological control of soil borne plant pathogens. During the last 25 years, they have emerged as the largest potentially most promising group of plant growth promoting rhizobacteria (PGPR) involved in the biocontrol of plant diseases. *Pseudomonas fluorescens* significantly reduced late leaf spot infection in groundnut when it was applied as seed treatment (Meena, 2000). Seed treatment followed by foliar application of *P. fluorescens* controlled the rice blast (Muthamilan, 1994), leaf spot and rust of groundnut and sunflower (Mathiyazhagan, 2003)

Bacillus species are the most common bacteria isolated from the soil, which account up to 36 per cent of the bacterial population. *Bacillus subtilis* is bestowed with the ability to endure stress, which is an advantage over other root colonizing bacteria that do not form endospores. This bacterium also has the ability to produce antifungal metabolites and antibiotics in soil. It can well be exploited for the control of many plant pathogens. The mechanism through which the antagonistic bacteria control the plant pathogens include the production of antifungal proteins, antibiotics, lytic enzymes etc., *B. subtilis* strain RB14 produced antibiotics like Iturin B and Surfactin which suppressed the damping off disease of

tomato (Asaka and Shoda, 1996). Antagonistic proteins isolated from *Bacillus* species could inhibit the growth of *Xanthomonas* sp, *Pseudomonas solanacearum*, *Pyricularia oryzae* and *R. solani* (Li Deba, 1997). Renuka (2003) reported that foliar application with *Bacillus* strains effectively controlled the *A. chlamydospora* in chrysanthemum.

2.4.1. Siderophore mediated competition for iron

Besides the capacity to colonize roots intensively for an extended period of time, the other mechanisms involved make the fluorescent pseudomonads as effective biocontrol agents.

Soil pseudomonads generally produce fluorescent, yellowish green, water-soluble siderophores of both hydroxymate and phenolate group. These siderophores have been classified as either pyoverdine or pseudobactin. The production of these siderophores has been linked with disease suppressing ability of certain species of fluorescent pseudomonads.

Siderophores are low molecular weight compounds that are produced under iron limiting condition, chelate the ferric ion (Fe^{3+}) with a high specific activity and serve as vehicle for the transport of Fe (III) into a microbial cell (Neilands, 1981). Siderophores produced by fluorescent pseudomonads that inhabit the plant rhizosphere have received much attention over the past decade, largely because of their proposed role in the biological control of soil borne plant pathogens and in disease suppressive soil (Leong, 1986; Loper, 1990). The fluorescent pseudomonads produce yellowish-green pigments that fluoresce under ultraviolet light and function as siderophore. The fluorescent siderophore termed as pyoverdine and pseudobactin represent one class of siderophores produced by the fluorescent pseudomonads (Demange *et al*., 1987; Teintze *et al*., 1981). Siderophore produced by *Pseudomonas* spp. was used in the control of *Cercospora* leaf spot of groundnut (Meena, 2000) and stem blight pathogen of *Phyllanthus amarus* (Mathiyazhagan, 2003).

2.4.2. Hydrogen Cyanide production (HCN)

Hydrogen cyanide production by certain fluorescent pseudomonads was found to influence the plant root pathogens. Suppression of black rot of tobacco (*Thielaviopsis bassicola*) by *P. fluorescens* CHA0 was mainly due to the production of HCN (Stutz *et al*., 1986). The effect of mutants of CHA0, deficient in HCN production was less than the parental strain to *T. bassicola* in tobacco.

Production of HCN by *P. fluorescens* strain E-11-3 was found to inhibit the mycelial growth of *Pythium in vitro* (Weststeijin, 1990), which was due to the influence of HCN on the pathogen or the host or both. Wei *et al*. (1991) reported that four PGPR strains of *P. fluorescens* G8-4, *P. aureofaciens* 28-9 and 36-5 and *P. putida* 34-13 produced HCN *in vitro*, whereas two strains *P. aureofaciens* 25-33 and *Serratia plymuthica* 2-67 that induced resistance in the host showed no HCN production.

2.5. Botanicals / Plant products

2.5.1. Antifungal properties of plant extracts

The presence of antimicrobial compounds in higher plants has long been recognized as an important factor to disease resistance (Mahadevan, 1982). Such compounds, being biodegradable and selective in their toxicity are considered valuable for controlling plant diseases (Singh and Dwivedi, 1987; Mason and Mathew, 1996; Kurucheve *et al.,* 1997; Amadioha, 2000 and Renuka, 2003).

2.5.2. Effect of plant products against foliar pathogens

Many workers have reported the presence of antimicrobial substances in plant extracts (Gerard Ezhilan *et al*., 1994 and Sateesh, 2001). Vijayan (1989) reported that bulb extract of *Allium sativum* effectively reduced the mycelial growth of *A. solani* in tomato. The inhibition of mycelial growth and spore germination might be due to the presence of antifungal components in the plant extracts. *Abutilon indicum* leaf extracts

reduced the mycelial growth of *A. alternata* (Sumathi, 1996). Thiribhuvanamala *et al.* (2001) reported that *Prosopis juliflora* and *Cocos nucifera* leaves significantly inhibited fungal spore germination and mycelial growth of *A. alternata*. *Acalypha indica* leaf extracts effectively controlled the mycelial growth of *A. alternata* (Bhowmic and Chaudhary, 1982). *A. indica* recorded maximum inhibitory effect against *A. alternata* (Meena and Marriappan, 1993).

Plant extracts of *Adenocallima alliaceaum, Azadirachta indica and Murraya koenigii* were effective against *A. alternata* (Rai *et al.*, 2000). Leaf extracts of *A. indica, M. koenigii, Citrus aurantifolia* and combination of *C. aurantifolia* and *A. indica* inhibited germination of *A. tenuissima* in pigeonpea (Singh *et al.*, 2001). Leaf extract of *Catharanthus roseus* and the rhizome extract of *Zingiber officinale* inhibited *A. triticina* in wheat (Shabana Parveen and Kumar, 2002). Narasimhan *et al*. (1995) reported that the leaf extracts of *Aegle marmelos* and *P. juliflora* inhibited the spore germination and mycelial growth of *A. tenuissima* causing blight of onion. Seed extract of *Glycine max* reduced the mycelial growth of *A. alternata* (Pandey *et al.*, 1982). Fewell and Rodick (1997) reported that α- chacomine, a glycoalkaloid from potato inhibited the spore germination of *A. brassicicola* and *Phoma medicaginis*. Plant extracts of *A. sativum, A. cepa, Curcuma longa, Z. officinale and A. indica* were effective against the Alternaria fruit rot of pomegranate (Jitendra Singh and Majumdar, 2001). Leaf blight of chrysanthemum caused by *A. chlamydospora* was effectively controlled by leaf extracts of *Abutilon indicum* and *Datura metel* (Renuka 2003). Similar observation have been made by Sinha and Saxena (1989), Sundariyal (1991), Rai *et al*. (2000) and Jitendra Singh and Majumdar (2001).

2.5.3. Active principles

Plant derivatives suppress the growth of plant pathogens by more than one mechanism. They are found to contain an array of chemicals and these chemicals are reported to induce various types of influence on the pathogens as well as on the host plant (Jayalakshmi, 1994). The active principles present in the plant products inhibit the spore germination and suppress the mycelial growth of the pathogens (Ansari, 1995; Kurucheve *et al*., 1997; Amadioha, 2000 and Renuka, 2003).

Last and Liewellyn (1997) studied the antifungal activities of many Australian native plants and isolated a 30 amino acid residue antifungal peptide from *Artiplex nummularia.* Aminoacid sequence analysis revealed its homogenity to the chitin binding antimicrobial peptides of *Amaranthus caudatus.* Dae Young *et al*. (1998) isolated and characterized a 4 kDa antimicrobial peptide from the seeds of *Phytolacca americana*, which was inhibitory to hyphal growth of *Botrytis cinerea* and *Pyricularia oryzae.*

2.6. Mass multiplication of biocontrol agents

Bacterial cell suspension cannot be used for large-scale field use due to its difficulty in storage, transport and handling. Hence a powder formulation with a longer shelf life would be beneficial. Addition of certain gums and polysaccharides as a sticker to the bacterial formulations did not reduce the viability of bacterial population (Suslow *et al*., 1979). Talc-based formulation of *P. fluorescens* effectively controlled the late leaf spot disease of groundnut with survival period of 90 days (Meena, 2000).

2.7. Methods of application

2.7.1. Seed bacterization

Seed bacterization refers to treating seeds with cultures that will improve plant growth that are also called as bacterial fertilizers. *Pseudomonas* spp. were not used extensively in bacterization studies, until recently when their potential as PGPR was demonstrated. Savithri and Gnanamanickam (1987) reported that seed bacterization of

peanut with *P. fluorescens* resulted in 59 per cent increase in yield and the root rot severity caused by *R. solani* was reduced by 55 per cent.

Seed bacterization with peat based formulation of *P. fluorescens* strain 1 at the rate of 10g kg^{-1} seed reduced rice blast and sheath blight (Muthamilan, 1994; Rabindran and Vidhyasekaran, 1996). The three-way mixture of PGPR strains INR7 (*B. pumulis*), SGBO3 (*B. subtilis*) and ME1 (*Curtobacterium flaccumfaciens*) as a seed treatment showed increased plant growth promotion and reduction of cucumber anthracnose caused by *Colletotrichum orbiculare*, angular leaf blight caused by *P. syringae* pv. *lachrymans* and wilt caused by *Erwinia tracheiphilia* (Raupach and Kloepper, 1998) under field conditions.

2.7.2. Foliar spray

The bacterium *P. fluorescens* was found to survive on the foliage when sprayed on the plants. Phyllosphere population of the bacterium was 2.3 X 10^4 cfu g^{-1} (Rabindran and Vidhyasekaran, 1996). Rice blast can effectively be controlled by foliar spray of talc based powder formulation of *P. fluorescens* strain Pf1 (1kg/ha). Late leaf spot disease in groundnut was effectively controlled by seed treatment and foliar application of *P. fluorescens* as talc based powder formulation (Meena, 2000).

2.8. Plant growth promotion

The bacteria that provide some benefit to plants are of two general types, those that form a symbiotic relationship with them and those that are free-living in the soil, but are often found near, on or even within the roots of plants. Free-living soil bacteria are usually referred to as PGPR. The beneficial effects of these bacteria have been variously attributed to their ability to produce phytohormones, organic acids and siderophores, to fix atmospheric nitrogen, to solubilize soil phosphate, which could be exploited for the biocontrol of plant diseases (Kloepper and Schroth, 1978).

Several strains of *P. fluorescens* increased the plant growth of rice and cotton by 27 and 40 per cent respectively when the bacterium was applied to seeds (Sakthivel *et al*., 1986; Lin *et al*., 1992). Dubeikovsky *et al*. (1993) suggested that indole acetic acid (IAA) production by *P. fluorescens* might influence the development of black currant cuttings and a stimulating IAA mediated effect of the bacteria on the development of the roots of the cuttings was observed. Increase in growth rate of rice plants by seed treatment with *P. fluorescens* was also reported (Muthamilan, 1994). Tosi and Zazzerini (1994) recorded an increase in length of sunflower seedlings by seed treatment with *P. fluorescens*. Significant plant growth promotion with increased runner length and leaf number per plant in cucumber by seed treatment and soil application of PGPR has been reported by Wei *et al*. (1996).

Williams and Asher (1996) achieved improvement in seedling emergence compared to seedlings from untreated seeds. Seed coating with pseudomonad isolates resulted in significantly greater root length, root and shoot biomass, pod yield and nodule number of groundnut compared with the control (Pal *et al*., 1999) and it was attributed to various factors such as ACC (1-amino cyclopropane) deaminase activity and siderophore production. Seed bacterization with *P. fluorescens* Pf1 strain improved root length and seedling emergence in groundnut (Meena, 2000).

Bacterization of seed with fluorescent pseudomonad GRC2 strain resulted in increased seed germination, early seedling growth, fresh nodule weight, grain yield and reduced charcoal rot disease incidence caused by *Macrophomina phaseolina* (Gupta *et al*., 2002). Bacterization of phyllanthus seeds with biocontrol agents against stem blight, increased the shoot and root length (Mathiyazhagan, 2003).

2.9. Induction of systemic resistance

The resistance can be broadly divided into local defenses that occur at the site of pathogen invasion and systemic defenses that are induced in uninoculated parts of the plant.

Induced protection in plants against various pathogens by biotic and abiotic inducers has been reported since 1930 when Chester (1933) proposed the term acquired physiological immunity. Since then several terms have been used to describe the phenomenon of induced resistance such as systemic acquired resistance (Ross, 1961), translocated resistance (Hurbert and Helton, 1967) and plant immunization (Tuzun and Kuc, 1991). The classical inducers of systemic resistance include pathogenic microorganisms like fungi, bacteria and viruses (Liu *et al*., 1995a), PGPR (Singh *et al*., 1990; Vidhyasekaran and Muthamilan, 1992) and incompatible pathogen interactions (Reimers *et al*., 1992).

Induced systemic resistance (ISR) by definition refers to protection of the plants systemically by enhancement of plant's defensive capacity against a broad spectrum of pathogens that is acquired after appropriate inducing agent upon infection by a pathogen. Induction of systemic resistance by PGPR against various diseases was considered as the most desirable approach in crop protection. First, the action of ISR is based on the defense mechanisms that are activated by inducing agents. Secondly, ISR once expressed activates multiple defense mechanisms that include increased activity of chitinases, β-1,3 glucanase and peroxidase (Xue *et al*., 1998; Dalisay and Kuc, 1995; Maurhofer *et al*., 1994; Schneider and Ullrich, 1994), accumulation of phytoalexins (van Peer and Schippers, 1992) and formation of protective biopolymers e.g. lignin, callose and hydroxyl proline-rich glycoproteins (Hammerschmidt and Kuc, 1982). Thirdly, an important aspect of ISR is a wide spectrum action against pathogens controlled by single inducing agent (Dean and Kuc, 1985; Hoffland *et al*., 1996; Wei *et al*., 1996).

Inoue *et al*. (1994) reported that treatment of susceptible barley leaves with papillae regulating extract from healthy leaves resulted in induction of systemic resistance against *Erysiphe graminis* f.sp. *hordei*. Renuka (2003) described the biochemical changes occurred in chrysanthemum plants due to the application of plant

products of *A. indicum* and *D. metel*. The PO, PPO and phenolic content were increased in Alternaria infected plants, when compared to healthy plants.

2.9.1. Mechanism of Induced systemic resistance

2.9.1.1. Phenylalanine ammonia - lyase (PAL)

PAL plays an important role in the biosynthesis of phenolics and phytoalexins (Daayf *et al*., 1997). The activation of the phenylpropanoid pathway in plants by environmental stimuli is one of the most universal biochemical stress responses known. PAL catalyzes the deamination of L-phenylalanine to transcinnamic acid, which is the first step in the biosynthesis of plant products such as lignin and phytoalexins. Induction of enzymes such as PAL, peroxidase and the accumulation of lignin can occur in response to insect and pathogen attack, exposure to oxidizing pollutants, mechanical stimulation and are thought to function in the resistance of plants to damage by these stresses. PAL is the key enzyme in inducing synthesis of salicylic acid (SA), which induces systemic resistance in many plants. Seed treatment and seedling root dipping with PGPR induced early and enhanced level of PAL in rice plants (Nayar, 1996). Plants treated with *Pseudomonas* strains had initially higher level of PAL, but these levels were lower in control (Chen *et al*., 2000).

2.9.1.2. Peroxidase (PO)

Enhanced activity of PO is often associated with resistance phenomenon such as lignin production. The enzyme is involved in the regulation of plant cell elongation, phenol oxidation, polysaccharide cross-linking, IAA oxidation, cross-linking of extension monomers and oxidation of hydroxyl-cinnamyl alcohols into free radical intermediates (Vidhyasekaran, 1997). Bradley *et al*. (1992) reported the increased activity of PO was correlated with resistance in many species including barley, cucurbits, cotton, tobacco, wheat and rice. It is also involved in polymerization of proteins and lignin or suberin

precursors into plant cell wall, thus constructing a physical barrier that prevents penetration of pathogen into host cell wall or movement through vessels.

Plant root colonization by PGPR was associated with PO activity (Albert and Anderson, 1987). Seed treatment and seedling dip induced early and enhanced levels of PO in rice plants (Nayar, 1996). The strains, PF1 and FP7 are the best inducers of plant chitinase and peroxidases, which are the most important compounds of ISR (Nandakumar *et al*., 2001). Foliar application of *Pseudomonas* and *Bacillus* strains induced the levels of PO in chrysanthemum caused by *A. chlamydospora* (Renuka, 2003).

2.9.1.3. Polyphenol oxidase (PPO)

Polyphenol oxidase usually accumulates upon wounding in plants. Biochemical approaches to understand PPO function and regulation are difficult because the reaction products of PPO covalently modify and cross-link the enzyme. The increased activation of PPO could be detected in cucumber leaf in the vicinity of lesions caused by some foliar pathogens. Induction of isoforms of PPO was noticed in *P. fluorescens* treated groundnut plants in response to infection by *C. personatum* (Meena. 2000).

2.9.1.4. Chitinase

Chitinase is a PR protein, which hydrolyzes chitin, a major cell wall component (3-10 %) of higher fungi. Chitinases cleave a bond between C_1 and C_4 of two consecutive N-acetyl glucosamine (GlcNAc) either by endolytic or exolytic mechanism. A large number of plant chitinases are of endochitinases type with molecular weight ranging from 25 to 36 kDa. The production of chitinase in plants has been suggested to be a part of their defense mechanism against fungal pathogens (Schlumbaum *et al*., 1986). Chitinase degrade the fungal cell wall chitin and inhibit the fungal growth at hyphal tip (Schlumbaum *et al*., 1986).

In recent years, several biocontrol agents like PGPR have been shown to induce systemic resistance in plants. Accumulation of chitinases and peroxidases with the onset of ISR by PGPR has been observed in some plants. Enhanced accumulation of chitinase in tobacco and bean leaves was observed in response to application of *Pseudomonas* spp. Increased chitinase activity in tobacco and maximum activity in cucumber have been observed as a result of systemic resistance by fluorescent pseudomonads against *P. syringae* pv. *tabaci* (Schneider and Ullrich, 1994). Nandakumar (1998) and Nayar (1996) found early and high induction of chitinase in *P. fluorescens* Pf1 treated rice plants, that offered protection against *R. solani* in rice.

2.9.1.5. β-1, 3 glucanase

Evidence of β-1, 3-glucanase in disease resistance was first reported by Kauffmann *et al*. (1987). In dicot plants, β-1,3-glucanase genes are considered to constitute a part of the general array of defense genes induced during pathogenesis (Boller, 1987; Mauch *et al*., 1988a). β-1,3-glucanase especially in conjunction with chitinase is capable of hydrolyzing fungal cell wall *in vitro* (Mauch *et al*., 1988b). Both of these enzymes are co-induced in response to fungal attack (Mauch *et al*., 1988a; Vogeli *et al*., 1988). Induction of β-1,3-glucanase was demonstrated in barley and other monocots like wheat, rice and sorghum in response to infection by *Bipolaris sorokiniana* (Jutidamrongphan *et al*., 1991).

2.9.1.6. Total phenols

Plant phenolics are the well-known antifungal, antibacterial and antiviral compounds and they play an important role in determining resistance or susceptibility of a host to parasite infection. Plant phenolics and their oxidation products such as quinones are highly toxic to invading fungi (Vidhyasekaran, 1988; Sequeira *et al*., 1991; Bennett and Wallsgrove, 1994; Southernton and Deverall, 1990; Cahill and Mc Comb, 1992). Bhaskaran (1976) reported that

total phenols were significantly low in necrotic tissue when compared to healthy in sunflower. Gupta *et al.* (1987) reported that the total phenol content was higher in disease resistant genotype of mustard as compared to susceptible ones. Chattopadhyay (1989) reported that the accumulation of phenols was greater in lesions than in lesion free areas of Alternaria infected leaves of rapeseed and mustard.

2.10. Serological diagnosis of plant disease by fungi

Immuno assay have been developed for a wide array of fungal plant pathogens (Dewey *et al*., 1991; Schots *et al.,* 1994). Some assays have been commercialized including kits for the detection of fungi belonging to the genera *Phytophthora*, *Pythium*, *Rhizoctonia* and *Septoria tritici*, *S. nodorum* and *Pseudocercosporella herpotrichoides* (Petersen *et al*., 1990; Smith *et al*., 1990; Mittermeier *et al.,* 1990; Miller and Joaquim, 1993). However, the kits have been useful for rapid field diagnosis (Ellis and Miller *et al.,* 1993) as an aid in making fungicides application decision (Timmer *et al.*, 1993), but are not routinely used in indexing programme. The types of fungal immunogens used include components of cell wall and spore surfaces, soluble antigens, broken cells and extra cellular antigens.

2.11. Epidemiology of the disease

Jefferey *et al.* (1984) reported that cultural debris serves as source of primary inoculum. Acimovic (1979) reported that the most favorable temperature for *A. helianthi* infection was 25^{o}C and 30^{o}C. Alternaria leaf spot in sunflower was more frequent and severe in areas with long wet summer together with daily mean temperature between 25^{o}C and 30^{o}C (Allen *et al*., 1983). High humidity is essential for infection (Frezzi *et al.,* 1979; Acimovic, 1979). The conidia need free water for germination to cause infection. Godoy and Ferades (1985) discussed the possibility of the disease to become the limiting factor to the sunflower culture expansion in Brazil especially in the

sowing from Septemper to December since at this time the conditions for the fungus development can be ideal. The disease development greatly favored by the average temperature between 24-27^{o}C (Abbas *et al*., 1995). Transmission studies showed that *A. alternata* is seed transmitted. In moist weather, these conidia germinate readily and putsforth 5-10 germ tubes from a single conidium. Germination of conidia occurs within 35 to 45 minutes at the optimum temperature of 28 to 30^{o}C (Sultana *et al.*, 1988). Incubation period varies from 48 to 72h (Singh, 1995). The dispersal of Alternaria conidia in the atmosphere of Badajoz city, Spain was studied using a Burkard spores trap, the seasonal and daily dispersal reached a maximum spores concentration level of 400-600 spores m^{-3} in 1993 to 1994 and concentration of 1700 spores m^{-3} in 1995. Daily periodicities of conidia showed two maximum peaks between 8 and 12 h and another between 19 and 20 h with a mainly diurnal pattern.

The weather conditions have greatest influence on the disease development especially total rainfall (Sentelhas *et al.*, 1996). Hervero and Zaldivar (1997) investigated daily levels of Alternaria spores in atmosphere in Palencia, Spain and reported that, it represented 55 per cent of all the identified spores. Their annual distribution pattern reached maximum in summer. Multiple regression analyses showed that concentration of Alternaria spores were positively correlated with maximum temperature and negatively correlated with the precipitation. Positive correlation was found for spore dispersal and temperatures and negative ones for north west winds, dew point, vapour pressure, rainfall, humidity, windless spells and South East winds (Paredes *et al.*, 1997).

CHAPTER III

MATERIALS AND METHODS

The groundnut *cv*. CO 2 was used throughout the experiment. The pot culture experiment was conducted in the glasshouse, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore and field experiments were carried out at Coconut Research Station (CRS), Aliyar Nagar.

3.1. Isolation of the pathogen

Groundnut leaves, showing typical leaf blight symptoms were collected. Tissues from the edge of the lesions were cut into 0.5 cm bits along with healthy tissues, surface sterilized with 0.1 per cent mercuric chloride, followed by three washings with sterile distilled water. Then, the tissues were placed on potato dextrose agar (PDA) medium. The pathogen was purified by hyphal tip method (Rangaswami, 1972) and maintained on PDA in slants at 4˚C.

3.2. Pathogenicity test

The pathogenicity of the fungus was proved by Koch's postulates, using the susceptible groundnut *cv*. CO 2. The plants were raised in the glasshouse in earthen pots (30 cm dia), filled with pot culture soil mixture. Conidial suspension of the pathogen was prepared according to Sutton and Shane (1983). Conidia were harvested from 20-day-old PDA culture by flooding the plates with sterile distilled water and then scrapping the agar culture three times to obtain sufficient inoculum. The conidial suspension was strained through two layers of cheesecloth and centrifuged at 4000 rpm for 10 min. to remove the nutrient supplied by the medium. Inoculum concentration was determined by using a haemocytometer and adjusted to 10^6 conidia^{-ml} with sterile distilled water. One ml teepol AG, (alkalylbenzene sodium salt) was added to 100 ml conidial suspension to increase the

spreading quality of the suspension. The conidial suspension was sprayed on the leaves of 30-day old groundnut plants maintained in the greenhouse, using an atomizer. Then, all inoculated plants were covered with polythene sheet up to five days to ensure high humidity. Symptom development was observed daily. The pathogen was re-isolated and compared with original isolate. The fungus was identified at Indian Type Culture Collection, IARI, New Delhi.

3.3. Survey for the incidence of *Alternaria alternata*

Survey was conducted during Rabi 2003 in major groundnut growing districts of Tamil Nadu *viz*., Coimbatore, Pudukkottai, Trichy, Thiruvannamalai, Cuddalore, Salem, Vilupuram, Madurai and Dharmapuri for the occurrence of the disease. In each district, one village with four fields were selected randomly. The disease intensity was assessed based on a 1-9 scale (Subrahmanyam *et al*., 1981) as described below.

Score	Disease description	Disease severity (%)
1	No disease	0
2	Lesion present largely on lower leaves, no defoliation	1-5
3	Lesions present largely on lower leaves, very few on middle leaves, defoliation of some leaflets evident on lower leaves.	6-10
4	Lesions present on all lower leaves and the middle leaves but severe on lower leaves; defoliation of some leaflets evident on lower leaves.	11-20
5	Lesions present on all lower leaves and middle leaves, over 50% defoliation on lower leaves	21-30
6	Severe lesions present on all lower leaves and middle leaves, lesions present but less severe on top leaves, extensive defoliation of lower leaves, defoliation of some leaflets evident on middle leaves.	31-40

Score	Disease description	Disease severity (%)
7	Lesion on all leaves but less severe on top leaves, defoliation of all lower and some middle leaves.	41-60
8	Defoliation of all lower and middle leaves, severe lesions on top leaves, some defoliation on top leaves evident	61-80
9	Almost all leaves defoliated, leaving bare stems, some leaflets may remain but show severe leaf spots	81-100

Per cent disease index was calculated by using the following formula.

$$\text{Per cent Disease Index (PDI)} = \frac{\text{Sum of individual rating}}{\text{No. of plants assessed}} \times \frac{100}{\text{Maximum disease category}}$$

3.4. Isolation of biocontrol agents

3.4.1. Isolation of *Pseudomonas* strains from the rhizosphere soil of groundnut

The plants were pulled out gently with roots intact and the excess soil adhering to the roots was removed gently. One gram of rhizosphere soil was transferred to 100 ml of sterile distilled water in a 250 ml Erlenmeyar flask. After thorough shaking, the different strains of *P. fluorescens* were isolated on King's B (KB) medium (King *et al*., 1954) by serial dilution plate method. One ml of bacterial suspension was poured in Petri dish containing King's B medium and incubated at 28 ± 2˚C for 24h. Colonies were viewed under UV light at 366 nm and the same was maintained in (KB) agar slants at 4°C. The Pf1 strain of *P. fluorescens* was obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. The other strains of *P. fluorescens* were isolated from groundnut plants, collected from various places of Tamil Nadu mentioned herein.

Pseudomonas strains isolated from the rhizosphere soil of different districts

Sl. No	***Pseudomonas* strains**	**Location / Districts**
1	*Pseudomonas fluorescens* –Pf2	Alangudi, Pudukkottai
2	*P. fluorescens* –Pf3	Vayalur, Trichy
3	*P. fluorescens* –Pf4	Velavaram, Thiruvannamalai
4	***P. fluorescens* –Pf5**	Valayammadevi, Cuddalore

3.4.2. Isolation of *Bacillus* sp. from the rhizosphere soil of groundnut

Bacillus spp. were isolated from the rhizosphere using nutrient agar (NA) as described earlier. *B. subtilis* strains isolated from the various places were designated as follows.

***Bacillus* strains isolated from the rhizosphere soil of different districts**

Sl. No	***Bacillus* strains**	**Location/ District**
1	*Bacillus subtilis* Bs1	Aliyar, Coimbatore
2	*B. subtilis* Bs2	Alangudi, Pudukkottai
3	*B. subtilis* Bs3	Vayalur, Trichy
4	*B. subtilis* Bs4	**Velavaram, Thiruvannamalai**
5	*B. subtilis* Bs5	Valayamadevi, Cuddalore
6	*B. subtilis* Bs6	Omalur, Salem
7	*B. subtilis* Bs7	Pichanatham, Villupuram
8	*B. subtilis* Bs8	Usilampatti, Madurai
9	*B. subtilis* Bs9	**Thimapuram, Dharmapuri**
10	*B. subtilis* Bs10	**Kalvettupalayam, Erode**

3.4.3. Preparation of bacterial inoculum

King's B and nutrient agar broths were inoculated with *Pseudomonas and Bacillus* strains, respectively. They were shaken constantly for 48 h at room temperature (28 ± 2˚C). Cells were removed by centrifugation at 8000 rpm for 10 min. at 4°C. Finally, an adequate amount of sterile distilled water was added until to obtain bacterial colonies of 10^8 cfu ml^{-1} (Thompson, 1996). In 600 ml of bacterial inoculum, 1 kg talc powder and 10 g carboxy methyl cellulose (CMC) was added. The groundnut seed was treated with the bacterial formulation at the rate of 10g kg^{-1} of seed.

3.5. *In vitro* screening of *Pseudomonas fluorescens* on radial mycelial growth of *A. alternata*

Pseudomonas fluorescens strains *viz*., Pf1, Pf2, Pf3, Pf4 and Pf5 were screened against mycelial growth of *A. alternata* by dual culture technique (Dennis and Webster, 1971). The bacterial culture was streaked on one side of the Petri dish (1 cm from the edge of the plate) containing PDA medium and mycelial disc (9 mm) of seven day-old culture of *A. alternata* was placed on the opposite side of the bacterial streak. Three replications were maintained for each treatment, in a randomized completed block design. The plates were incubated at room temperature (28 $\pm$ 2°C) and the zone of inhibition was measured. The mycelial growth of the pathogen and per cent reduction over control was calculated by using the formula.

$$\text{Per cent reduction over control} = \frac{\mathbf{C - T}}{C} \times 100$$

where,

C – mycelial growth of pathogen in control

T – mycelial growth of pathogen in dual plate

3.6. *In vitro* screening of *B. subtilis* strains on radial mycelial growth of *A. alternata*

The *Bacillus* strains *viz*., Bs1, Bs2, Bs3, Bs4, Bs5, Bs6, Bs7, Bs8, Bs9 and Bs10 were screened against radial mycelial growth of *A. alternata* in dual culture technique, as described earlier.

3.7. *In vitro* screening of plant extracts against *A. alternata*

3.7.1. Preparation of Plant extracts

The presence of antifungal principles (AFPs) in leaf extracts of various plant species were tested. The extracts were prepared by grinding the leaves in a mortar-using pestle by adding water at the rate of 10 ml g^{-1} of plant tissue and filtered through thin layer of cotton wool. The plant extract was diluted further to have 10 per cent concentration (v/v).

3.7.2. *In vitro* evaluation of plant extracts against *A. alternata*

Leaf extracts of following plant species were tested for their efficacy against *A. alternata.*

Sl. No	Scientific name	Common/Vernacular name	Family
1.	*Abutilon indicum L.*	Thuthi	Malvaceae
2.	*Azadirachta indica L.*	Neem	Meliaceae
3.	*Bougainvillea spectablis L.*	**Bougainvillea**	Nyctaginaceae
4.	*Catharanthus roseus L*	Periwinkle	Apocynaceae
5.	*Acalypha indica*	Kuppaimeni	Acanthaceae
6.	*Ocimum sanctum*	Tulsi	Labiatae
7.	*Lawsonia inermis*	Maruthani	Sonneratiaceae
8.	*Datura metel*	Umathai	Solanaceae
9.	*Prosopis juliflora*	Velikaruvel	Mimoceae
10.	*Tridox procumbens*	Vettukayapoondu	Compositae

Sl. No	Scientific name	Common/Vernacular name	Family
11.	*Cyprus rotandus*	**Koraipul**	Cyperaceae
12.	*Vitex negundo*	Notchi	Verbanaceae
13.	*Ipomea cornea*	Velikattamanakku	Convolvulaceae
14.	*Cynadon dactylon*	Haryali	Graminae
15.	*Pongamia glabra*	Pungam	Leguminaceae
16.	*Neerium indicum*	Arali	Apocynaceae

Poisoned food technique (Schmitz, 1930) was followed to study the antifungal activity of various plant extracts. Ten ml of respective plant extract (100 %) was mixed with 90 ml of PDA to obtain 10 per cent concentration and sterilized at 15 lbs pressure for 30 min. Twenty ml of amended medium was poured into sterile Petri plates and 9 mm mycelial disc of the test fungus *A. alternata* was placed on the centre of the plate. They were incubated at room temperature (28 ± 2^{o}C). Three replications were maintained for each treatment in a randomized complete block design. PDA without leaf extract having sterile distilled water served as control. Per cent reduction of mycelial growth over control was calculated.

$$\text{Per cent reduction over control} = \frac{C - T}{C} \times 100$$

where,

C – mycelial growth of pathogen in control

T – mycelial growth of pathogen in poisoned media

3.8. Plant growth promotion by biocontrol agents

The plant growth promoting activity of biocontrol agents was assessed based on seedling vigour index (ISTA, 1966). The germination test was conducted in sand

medium, using 50 groundnut seeds taken at random. Eight replications were maintained in a randomized complete block design. The temperature of the germination chamber was maintained at 25 ± 2^{o} C with RH of 90 ± 3 per cent. Per cent of seed germination, root length and shoot length of individual seedlings were measured. The vigour index was calculated by using the formula described by Abdul Bagi and Anderson (1975).

Vigour index = (Mean root length + Mean shoot length) x Germination (%)

3.9. Bacterial metabolites

3.9.1. Salicylic acid production

Salicylic acid production of the Pf strains was determined as per the method of Meyer *et al*. (1992). The bacterial strains were grown in the standard succinate medium (Succinic acid- 4.0g, K_2HPO_4-6.0g, KH_2PO_4 3.0g, $(NH_4)_2SO_4$ 1.0g, $MgSO_4$ $7H_2O$-0.2g, distilled water-1000ml, pH 7.0) at 28 ± 2°C for 48 h. Cells were collected by centrifugation at 6000 rpm for 5min. Four ml of cell free culture filtrate was acidified with 1N HCl to pH 2.0 and salicylic acid was extracted with equal volume of chloroform. Four ml of water and 5µl of 2M $FeCl_3$ were added to the pooled chloroform phases. The absorbance of the purple iron salicylic acid complex, which was developed in the aqueous phase was read at 527nm. Salicylic acid was measured according to a standard curve drawn with salicylic acid dissolved in succinate medium and treated as described above. The quantity of salicylic acid was expressed as μgml^{-1} of the culture filtrate.

3.9.2. Siderophore production

The strains of *P. fluorescens* were grown in KB broth for 3 days and centrifuged at 2000 rpm for 10 min. The pH of supernatant was adjusted to 2.0 with HCl and equal quantity of ethyl acetate was added into separating funnel, mixed well and ethyl acetate fraction was collected. This was repeated three times to bring the entire quantity of

siderophore from the supernatant. The ethyl acetate fractions were pooled, air-dried and dissolved in 5 ml of ethanol (50%). Five ml of ethyl acetate fraction was reacted with 5 ml of Hathway's reagent (1.0 ml of 0.1 M $FeCl_3$ in 0.1 N HCl to 100 ml of distilled water + 1.0 ml of potassium ferriccyanide). The absorbance of dihydroxy phenol was read at 700 nm. A standard curve was prepared using dihydroxy benzoic acid (Dileep *et al*., 1998). The quantity of siderophore synthesized was expressed as μg ml^{-1} of culture filtrate.

3.9.3. Indole Acetic Acid (IAA) production

Production of indole acetic acid (IAA) by *P. fluorescens* in the medium was assayed by the method described by Gorden and Paleg (1957). The *P. fluorescens* was grown in triptic soy broth (TSB) (animal peptone 15.0 g; Soya peptone 5.0 g; NaCl 5.0g; glycine 4.4g; distilled water 1000 ml) with tryptophan as a precursor (100 μg / ml) and incubated in a rotary shaker for 30 h. Supernatant from the cultures were collected after centrifugation at 2000 rpm for 10 min. To one ml of cell free culture filtrate, 2 ml Salkowsky reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HclO_4$) was added and incubated at 28 ± 2°C for 30 min. The absorbance was measured at 530 nm. A standard curve was prepared with IAA and quantity of IAA produced was expressed as μg ml^{-1}.

3.9.4. Hydrogen cyanide (HCN) production

3.9.4.1. Qualitative assay

Production of HCN was determined by the method described by Miller and Higgins (1970). Bacteria were grown on tryptic soy agar (TSA). A sterile filter paper disc of 1.5cm was soaked in picric acid solution (2.5 g of picric acid, 12.5 g of Na_2 CO_3, 1000 ml of distilled water) and placed in the upper lid of each Petri dish. Dishes were sealed with parafilm and incubated at 28^{o}C for four days. Hydrogen cyanide production was

assessed by the change of light yellow colour of filter paper to brown or reddish brown as an indication of weak, moderate or strong production of HCN by each strain.

3.9.4.2. Quantitative assay

Bacteria were cultured on tryptic soy broth (TSB) in sterile conical flasks. Filter paper was cut into uniform strips of 10 cm length and 0.5 cm wide, saturated with alkaline picrate solution and a single filter paper was placed inside the each conical flask in a hanging position. After incubation at 28 ± 2°C for 48 h, the sodium picrate in the filter paper was reduced to reddish compound in proportion to the amount of hydrocyanic acid evolved. The filter paper was placed in a clean test tube containing 10 ml of distilled water and the colour was eluted and absorbance was measured at 625 nm (Sadasivam and Manickam, 1992).

3.10. Estimation of biochemical constituents of plant products

3.10.1. Phenolic content

The phenolic content of leaf samples *viz., A. indicum, D. metel, L. inermis* and *A. indica* was determined as per the procedure given by Zieslin and Ben-Zaken (1993). One gram of the tissue was homogenized in 10 ml of methanol (80%) and agitated for 15 min. at 70^{o}C. One ml of the methanolic extract after evaporation was added to 5 ml of distilled water and 250 ml of Folin Ciocalteau reagent (1 N) was added and the solution was kept at 25^{o}C. After three min, one ml of saturated solution of $Na_2 CO_3$ and one ml of distilled water was added and the reaction mixture was incubated for 1h at 25^{o}C. The absorbance of the developed blue colour was measured using a Hitachi 200-20 Spectrophotometer at 725 nm. The content of total soluble phenols was calculated according to a standard curve obtained from a Folin – Ciocalteau reaction with a phenol solution (C_6H_6O) and expressed as phenol equivalents in mg g^{-1} fresh tissue.

3.10.2. Protein content

Protein was estimated by the method of Bradford (1976). Ten mg of Coomassie brilliant blue G-250 was dissolved in 4.7 ml of absolute alcohol and 10 ml of concentrated phosphoric acid and the volume was made up to 100 ml with distilled water. A sample of 50 µl was added to 950 µl of dye solution and the mixture was incubated for 5 min at room temperature. The absorbance was recorded at 595 nm in Hitachi 200-20 Spectrophotometer. Bovine serum albumin was used as standard.

3.10.3. Purification of proteins

Ten gram of leaf sample was ground with 20 ml of sodium phosphate buffer (pH 7.0), filtered and centrifuged at 10,000 rpm for 20 min at 4^{o}C. The supernatant was then precipitated with 80 per cent saturated ammonium sulphate and a pellet was obtained after centrifugation further at 12,000 rpm for 20 min. The pellet was resuspended in minimal amount of distilled water and finally dialyzed against 70 mM potassium phosphate buffer, pH 6.0.

3.11. Thin Layer Chromatography (TLC)

3.11.1. Preparation and activation of TLC plates

Silica Gel-G (E-Merck) was coated on TLC (20x20 cm) plate to form thin layer. Twenty gram of fine silica gel-G powder was mixed thoroughly with 40 ml of distilled water and applied to TLC plate by using applicator, to form 0.5 mm thickness. A fine layer of 0.5 mm thick wet silica gel was formed on the glass plate. The glass plate was allowed to dry in open air for 1 h and then in hot air oven at 110^{o}C for 2 h. The activated TLC plate was applied with leaf samples (20 µl) with the help of micropipette.

3.11.2. Separation of compounds by TLC

The solvent system used was chloroform : methanol (90:10) for phenolic compounds and (15:5) for alkaloids. The TLC plate was slowly placed into the tank so as to immerse 0.5 cm into the solvent at the bottom. The tank was closed with a glass lid so as to have the chamber completely filled with the solvent vapours. When the solvent front reached the end of the plate, the plate was removed from the tank and kept in open air at room temperature to evaporate the solvent.

3.11.3. Identification of compounds

The dried TLC plate was sprayed and exposed to various reagents so as to identify the compounds present in the leaf extract.

3.11.3.1. Spray reagents

3.11.3.1.1 Dragendorff's reagent

Dragendorff's reagent was used to identify the presence of alkaloids.

Solution A: 1.7 g bismuth nitrate was dissolved in 100 ml of water: acetic acid (8:2)
Solution B: 40 g of potassium iodide was dissolved in 100 ml water.
Solution C: 20 ml of acetic acid was mixed with 70 ml water.

Finally 5 ml of A and 5 ml of B were added to the solution C to make up to 100 ml just before use. Dried plate was sprayed with the above solution. Presence of red, orange, brown and yellow spots indicated the presence of alkaloids.

3.11.3.1.2. Diazotized sulphanilic acid solution

Fifty mg of diazotized sulphanilic acid was added to 20 ml of 20 per cent Na_2CO_3 solution and sprayed on the dried TLC plates. Yellow, orange and red coloration indicated the presence of phenols.

Diazotized sulphanilic acid was prepared by dissolving 25 g of sulphanilic acid in 125 ml sodium nitrate (10 %) solution. This mixture was added drop by drop to 60 ml 8 M HCl under ice-cold condition. After 10 min, this mixture was filtered in cold water and then washed with ethanol and ether repeatedly. The crystals were air dried and stored at 4^{o}C.

3.11.3.1.3. Folin-Ciocalteau reagent

This reagent was used for identification of phenols. Folin- Ciocalteau reagent was diluted (1:1) with water and sprayed on the dried TLC plate followed by 20 per cent Na_2CO_3 solution. Presence of blue spots indicated the presence of phenolic compounds.

3.11.4. Isolation of TLC separated compounds

The TLC technique was also used for isolation of separated compounds and in that case it is called as preparative TLC. Instead of thin layer, a thick layer (2mm) of absorbent (silica gel) was coated and a greater quantity of the sample was applied onto the plate as a streak rather than a spot. After separation, the area of the separated compound was scraped off, eluted with methanol and recovered in a relatively pure form.

3.11.5. Antifungal activity of separated compounds

To test the antifungal activity of the separated compounds, PDA medium was melted, cooled and then 10 ml of the medium was poured into the sterilized Petri plates and allowed to solidify. Three wells were made in solidified media near the periphery of the plate. Later nine mm mycelial disc of *A. alternata* was kept in the middle of the Petri dish. The wells were filled with the partially purified bioactive compounds and incubated at room temperature (28 ± 2^{o} C) for eight days. After incubation period, the plates were examined for the inhibition of the mycelial growth.

3.12. Induction of defense mechanism against *A. alternata*

The promising biocontrol agents *viz*., Pf1, Pf2, Bs1, Bs 5, and plant products of *A. indicum* and *D. metel* effective under pot culture condition were selected for testing their activity for the induction of defense mechanism. The experiment consisted of the following treatments.

Treatment details

T1	ST+FS (Pf1)
T2	ST+FS (Pf1+PI)
T3	ST+FS (Pf2)
T4	ST+FS (Pf2+PI)
T5	ST+FS (Bs1)
T6	ST+FS (Bs1+PI)
T7	ST+FS (Bs5)
T8	ST+FS (Bs5+PI)
T9	*A. indicum* (10 %)
T10	*A. indicum*+PI
T11	*D. metel* (10 %)
T12	*D. metel* + PI
T13	Inoculated control
T14	Healthy

ST – Seed treatment – 10g/kg, FS - Foliar spray - 0.2%, PI-Pathogen inoculation

The seeds were treated with talc based formulation of biocontrol agents @ 10 g kg^{-1} of seed. Thirty days after sowing, the plants were sprayed with the biocontrol agents @ 0.2 per cent concentration and leaf extracts @ 10 per cent concentration

separately. The plants were challenge inoculated with the pathogen one-day after the spray. Plants inoculated with the pathogen and uninoculated healthy were also maintained. In each treatment three replications were maintained in a randomized complete block design.

The groundnut leaves were collected from the treated plants at different intervals *viz*., 1, 3, 5 and 7 days after challenge inoculation with the pathogen and used for the analysis of defense related enzymes.

One gram of leaf tissue was homogenized with 1 ml of sodium phosphate buffer (pH 7.0). Homogenized samples were centrifuged at 10,000 rpm for 10 min. The supernatant solution was used as source for analyzing phenylalanine ammonia-lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), β-1, 3 glucanase, chitinase, tyrosine ammonia lyase (TAL), protein and phenol.

3.12.1. Assay of Phenylalanine Ammonia Lyase (PAL) activity

Leaf samples (1 g) were homogenized in 3 ml of ice cold 0.1 M sodium borate buffer, pH 7.0 containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinyl pyrrolidone. The extract was filtered through cheese cloth and the filtrate was centrifuged at 16,000 rpm for 15 min. The supernatant was used as enzyme source. Phenylalanine ammonia lyase activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm as described by Dickerson *et al.* (1984). Sample containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.l M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30^{o}C. The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient of 9630 M^{-1}. Enzyme activity was expressed as nmol trans-cinnamic acid min^{-1} mg^{-1} of protein.

3.12.2. Assay of Peroxidase activity (PO)

One gram of fresh leaf tissue was ground with 2 ml of 0.1M phosphate buffer, pH 7.0 at 4^{o}C. The homogenate was centrifuged at 6000 g at 4^{o} C for 10 min. and the supernatant was used as enzyme source. The reaction mixture consisted of 1.5 ml of 0.05M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of one per cent H_2O_2. The reaction mixture was incubated at room temperature (28 ± 2°C). The change in absorbance was recorded at 420 nm at 30 sec interval for 3 min. The boiled enzyme preparation served as blank. The enzyme activity was expressed as changes in the absorbance of the reaction mixture min^{-1} mg^{-1} of protein (Hammerschmidt *et al.,* 1982).

3.12.3. Assay of Polyphenol oxidase activity (PPO)

Polyphenol oxidase activity was determined as per the procedure given by Mayer *et al.* (1965). One gram of leaf sample was homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 16,000 rpm for 10 min at 4°C and the supernatant was used as the enzyme source. The reaction mixture consisted of 200 µl of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and to start the reaction, 200 µl of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm min^{-1} mg^{-1} of protein.

3.12.4. Assay of Chitinase

The colorimetric assay of chitinase was carried out according to the procedure developed by Boller and Mauch (1988).

a. Preparation of colloidal chitin

Colloidal chitin was prepared by treating 1 g of crab-shell chitin powder with acetone to form a paste and 20 ml of concentrated hydrochloric acid (HCl) was added slowly while grinding in a mortar with the temperature maintained at 5^{o}C. After several minutes the content was then filtered through glass wool and collected in ice cold ethanol by constant stirring to precipitate the chitin in a highly dispersed state. The residue was

sedimented and resuspended in distilled water several times to remove excess acid and alcohol and then dialyzed against tap water. Chitin content of the suspension was determined by drying a sample *in vacuo* and adjusted with distilled water to a final concentration of 10 mg ml^{-1} (dry weight/ volume) and stored at $5^{o}C$ (Berger and Reynolds, 1958).

b. Snail gut enzyme

Six hundred mg of the commercial lyophilized snail gut enzyme (Helicase, obtained from Sepracor, France) was dissolved in 10 ml of 20 mM potassium chloride (KCl) and chromatographed on a Sephadex G-25 column (38 x 1.5 cm) using a 10 mM KCl solution, containing 1mM EDTA and adjusted to pH 6.8 for equilibration and elution. The first 20 ml was eluted after the void volume collected (Boller and Mauch, 1988).

c. Preparation of p-dimethylaminobenzaldehyde (DMAB) reagent

The DMAB reagent was prepared by the procedure described by Reissig *et al.* (1959). Stock solution of DMAB was prepared by mixing 8g of DMAB in 70 ml of glacial acetic acid along with 20 ml of concentrated HCl. One volume of stock solution was mixed with 9 volumes of glacial acetic acid immediately before use.

d. Enzyme extraction

One gram of groundnut leaves were extracted with 5 ml of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 10,000 rpm and the supernatant was used as an enzyme source.

e. Assay procedure

The assay mixture consisted of 10 µl of 1 M sodium acetate buffer (pH 4.0), 0.4 ml of enzyme extract and 0.1 ml of colloidal chitin (0.1%). After 2 h of incubation at 37^{o}C, the reaction was stopped by centrifugation at 1000 g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted out and transferred into a glass reagent tube containing 30 ml of 1 M potassium phosphate buffer (pH 7.1) and incubated with 20 µl of (w/v) desalted snail gut enzyme (3%) (w/v) for 1 h. After 1 h, the pH of the reaction mixture was brought to 8.9 by the addition of 70 µl of 1 ml sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min and then rapidly cooled in an ice water bath. After addition of 2 ml of DMAB reagent, the mixture was incubated for 20 min at 37^{o}C. Immediately thereafter, the absorbance value at 585 nm was measured using a Hitachi 200-20 Spectrophotometer. N-acetyl glucosamine (GlcNAc) was used as standard (Boller and Mauch, 1988). The enzyme activity was expressed as nmol GlcNAc min^{-1} mg^{-1} fresh tissue.

3.12.5. Assay of β-1, 3- glucanase

β-1, 3- glucanase was analyzed by the laminarin-dinitro salicylic acid method (Pan *et al*., 1991). The reaction mixture consisted of crude enzyme extract of 62.5 µl and 62.5 µl of 4% laminarin. The reaction was carried out at 4° C for 10 min. and the reaction was stopped by adding 375 µl of dinitro salicylic acid and heated for 5 min. on a boiling water bath. The resulting solution was diluted with 4.5 ml distilled water and the absorbance was read at 500 nm. The crude extract preparation with laminarin at zero time incubation served as blank. The enzyme activity was expressed as µg equivalent of glucose released min^{-1} mg^{-1} of protein.

3.12.6. Assay of Tyrosine Ammonia Lyase activity (TAL)

One gram of leaf sample was homogenized in 3 ml of 0.1 M sodium borate buffer (pH 7.0) containing 30 µg insoluble polyvinyl pyrolidone (PVP). The homogenate was filtered through four layers of cheese cloth. The filtrate was centrifuged at 20,000 g for 30 min at 4^{o}C. The supernatant served as the source of enzyme extract. The reaction mixture consisted of 50 µl enzyme extract, 2.45 ml of 1M sodium borate buffer (pH 8.8) and 0.5 ml of 2mM tyrosine. The absorbance values were read spectrophotometrically at 290 nm. The TAL activity was expressed as change in absorbance (in units) min^{-1} g^{-1} fresh tissue (Dickerson *et al*., 1984).

3.12.7. Phenolic content

The phenolic content of the groundnut leaf sample was determined as per the procedure given in the section 3.10.1.

3.12.8. Protein

Protein content of the groundnut leaf sample was determined by Bradford's method as given in the section 3.10.2.

3.13. Native polyacrylamide gel electrophoretic analysis (Native PAGE)

The isoform profile of PO and PPO were examined by discontinuous native poly acrylamide gel electrophoresis analysis (Native PAGE) (Laemmli, 1970). Leaf samples showing maximum activity of PO and PPO in spectrophotometer assay were used for analysis. Induction of defense reaction against *A. alternata* in groundnut was tested for the activity of PO and PPO and was found to be maximum on 5th day after challenge inoculation. Hence leaf samples collected on 5th day after challenge inoculation with *A. alternata* were used for the isozyme analysis.

3.13.1. Isozymes

Seed treatment and foliar spray with biocontrol formulation and plant products were done as described earlier. One set of 30 days old plants treated with the biocontrol agents Pf1, Pf2, Bs1 and Bs5 while another set of treated plants were challenge inoculated with the pathogen. Healthy and inoculated plants were also maintained. After five days of inoculation with the pathogen, samples were collected and analyzed for the induction of isozyme.

3.13.1.1. Peroxidase isozyme (PO)

Groundnut leaf samples were homogenized in 0.1 M sodium phosphate buffer pH 6.0 (2 ml/g fresh tissue) (Smith and Hammerschmidt, 1988). Peroxidase isozymes were analyzed as per the procedure given by Pan *et al.* (1991) by native polyacrylamide gel electrophoresis (PAGE). The gels were run for 8 h at 10^{o}C (constant current: 30 mA) with a Hoefer vertical electrophoresis unit. Samples of 100 µl containing 1 µg protein were electrophoresed at 30 mA for 6 h. Gel was stained for peroxidase with 0.2 M acetate buffer at pH 4.5 containing benzidine (0.05%) and H_2O_2 (0.03%). After staining, the gel was immersed in acetic acid (7%) for 3 min and washed with distilled water (Nadlony and Sequeira, 1980).

3.13.1.2. Polyphenol oxidase isozymes (PPO)

The PPO was assayed by homogenizing 1 g of tissue in 0.01 M potassium phosphate buffer (pH 7.0) containing non-ionic detergent (Tween 20) (1%) at 4^{o}C for 15 minutes. The homogenate was centrifuged at 20,000 g for 15 min at 4^{o}C in a refrigerated centrifuge and the supernatant was used as the enzyme source. After native PAGE, the gel was equilibrated for 30 min in p-phenylene diamine (0.1%) in 0.1 M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer.

The addition of catechol was followed by a gentle shaking which resulted in appearance of dark brown discrete protein bands (Jayaraman *et al.,* 1987).

3.14. Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

One gram of leaf sample was ground in a chilled pestle and mortar with 2 ml of ice-cold potassium phosphate buffer (0.1M) at pH 6.5. The buffer extracts were then centrifuged at 10,000 rpm for 20 min. at 4^{o}C and the supernatant was used for the SDS-PAGE. The protein content of the sample was determined by the procedure as described by Bradford (1976).

Hundred µl of protein from treatments were taken and mixed with 10 µl of sample buffer in microfuge tube, boiled for 4 min. and incubated at 4^{o} C for 30 min. Then the samples containing equal amount of proteins were loaded in to the wells of polyacrylamide gels (Annexure) (Sigma Aldrich Techware System, Sigma, USA). The medium molecular weight markers (GENEI, Bangalore) were used. Electrophoresis was carried out at constant voltage of 75V. The gels were stained with Coomassie brilliant blue (G 250) solution based on the Rf value of each protein band stained, the molecular weight was calculated.

3.15. Screening of biocontrol agents and plant products against *A. alternata* under green house condition

The promising biocontrol agents (Pf1, Pf2, Bs1 and Bs5) and the plant products (*A. indicum* and *D. metel*) under *in vitro* condition, were further tested against *A. alternata* under greenhouse condition. The treatments were given as seed treatment (ST), seed treatment plus foliar application (ST+FS) and foliar spray (FS) alone as described below.

Treatment details

T1	Pf1 (ST)
T2	Pf1 (ST+FS)
T3	Pf1 (FS)
T4	Pf2 (ST)
T5	Pf2 (ST+FS)
T6	Pf2 (FS)
T7	Bs1 (ST)
T8	Bs1 (ST+FS)
T9	Bs1 (FS)
T10	Bs5 (ST)
T11	Bs5 (ST+FS)
T12	Bs5 (FS)
T13	*A. indicum* (10%) (FS)
T14	*D. metel* (10%) (FS)
T15	Mancozeb 75 % WP (0.2%)- (FS)
T16	Untreated control

ST - Seed treatment - 10g /kg, FS-Foliar spray - 0.2 per cent

Foliar sprayings were given at fortnightly intervals, starting from 30 days after sowing. The observation on disease intensity was recorded at 90 DAS on a 1-9 scale as described earlier. Various parameters like plant height and number of branches were also recorded at 30, 45 and 70 DAS. At the time of harvest, yield and biomass production were recorded.

3.16. Efficacy of promising biocontrol agents and botanicals against groundnut leaf blight disease (*A. alternata*), under field conditions

Field experiment were conducted at CRS, Aliyar, to evaluate the promising (greenhouse) biocontrol agents of Pf1, Pf2, Bs1, Bs 5 and plant products *viz., A. indicum* and *D. metel* against *A. alternata.* The field trials were laid out with eight treatments replicated thrice in a randomized complete block design, using a plot size of 4x3m. The observations on disease intensity was recorded at 90 DAS, on a 1-9 scale as described earlier. The yield and total biomass production were recorded during harvest.

Treatment details

T1	Pf1 (ST+FS)
T2	Pf2 (ST+FS)
T3	Bs1 (ST+FS)
T4	Bs5 (ST+FS)
T5	*A. indicum* 10%
T6	*D. metel* 10%
T7	Mancozeb 75 % WP (0.2%) (FS)
T8	Untreated control

ST- Seed treatment at 10 g / kg.

FS- Two foliar sprays at fortnightly intervals starting from 30 DAS.

3.17. Detection of *A. alternata* by Enzyme Linked Immunosorbent Assay (ELISA)

3.17.1. Raising of Polyclonal antibodies

A. alternata was grown on PDA broth for 15 days. Freshly harvested mycelial mat was ground in 0.1 M phosphate buffer (pH 7.0) and clarified at 12,000 rpm for 10 min at room temperature. One ml of the antigen was mixed with one ml of Freund's complete adjuvant, saline and emulsified in a cyclomixer. The emulsion was given intramuscularly (4 months old New Zealand white rabbits weighing about

2.5 kg) with sterile syringe for production of PAbs (polyclonal antibodies) against *A. alternata* mycelial protein, after adjusting the protein content to 150 g ml^{-1} (Bradford, 1976). Four injections were given at weekly intervals however, the fourth injection was an incomplete adjuvant. Bleeding was done 15 days after the last injection and the blood was transferred to sterile glass vials and allowed to stand in a slanting position until coagulated. The antiserum was transferred to sterile centrifuge tubes and the red blood cells were pelleted by centrifugation (10,000 rpm, 4°C, 10 min) three times. The antiserum was stored at 4°C for further studies.

3.17.2. Antigen preparation

Leaf samples were collected from both healthy and infected plants. After washing, the samples were ground in 0.1 M Carbonate buffer (pH 9.6) in a sterile pestle and mortar at room temperature (28 $\pm$ 2°C) and clarified at 12,000 rpm for 10 min. at 4°C. The supernatant was stored at 4°C for further studies.

3.17.3. Direct Antigent Coating (DAC) ELISA

DAC-ELISA method (Clark and Adams, 1977) was used for detection of *A. alternata*. Microtitre plates (Tarson, India) were first coated with 100 µl of antigen for 2 h at 37°C. The plates were washed three times with PBS-T. The primary antibodies diluted in PBS-T (1:1000) containing two per cent polyvinyl pyrrolidone and 0.2 per cent ovalbumin (PBS-TPO) were added (100 µl per well) separately. After incubation at 37°C (2 h), the plates were washed using PBS-T. Alkaline phosphatase (ALP) conjugated goat anti-rabbit immunoglobulin (Bangalore Genei, India) (1:6000 with PBS-TPO) were added @ 100 µl per well. The plates were incubated for 2 h at 37°C. After washing in PBS-T, 100 µl of 4-nitrophenyl phosphate (SD Fine chemicals, India) dissolved in diethanolamine at 1 mg ml^{-1} (Sigma, USA) (pH 9.8) was added. The colour development (absorbance) was read at 405

nm with a microplate reader (Bio Rad Model 3550, USA). The reaction was terminated by adding 50 µl of 3 M NaOH after incubation for half an hour at room temperature (28 $\pm$ 2°C).

3.18. Studies on the role of weather parameters on the incidence of *A. alternata*

Three field trials were conducted to find out the effect of various weather parameters on disease incidence, during Rabi 2002-'03, Kharif 2003-'04 and Rabi 2003-'04 at CRS, Aliyar Nagar. For each season, three sowings were taken at fortnightly intervals with two treatments *viz.*, insect pest control and control plot without any treatment. The insect pest control treatment was maintained to keep the plants free from insect pest infestation. A plot size of 120 square metres was adopted for each treatment. The susceptible groundnut *cv*. CO 2 was sown with a spacing of 30x10 cm. Disease intensity was assessed at weekly intervals, after onset of the disease. For observation, ten plants were selected randomly, tagged and in each plant 3 trifoliate leaves *viz*., upper, middle and lower leaves were selected. The disease intensity was recorded based on 1-9 scale as described earlier.

Various direct weather parameters *viz*., maximum and minimum temperature, relative humidity, rainfall and bright sunshine hours and the derived weather parameters *viz*., Growing Degree Days (GDD), Diurnal Variation (DV) and Relative Temperature Disparity (RTD) were recorded and correlated with disease intensity.

where,

$$\mathbf{DV = Maximum\ temperature - Minimum\ temperature\ of\ a\ day.}$$

$$RTD = \frac{\text{Maximum temperature - Minimum temperature}}{\text{Maximum temperature}} \times 100$$

$$GDD = \frac{\left(\text{Maximum temperature} + \text{Minimum temperature} \right)}{2} - \text{Base temperature}$$

where,

Base temperature for groundnut = $18^{o}C$

For correlation and regression analysis, the average weather parameters that prevailed before 10 days of observation were recorded based on the time taken for symptom development. The correlation and multiple regression analysis were done by using MSTAT software.

3.19. Statistical analysis

The statistical analysis of the data was made using package developed by IRRISTAT version 92 of International Rice Research Institute Biometrics Unit, The Philippines.

CHAPTER III

MATERIALS AND METHODS

The groundnut *cv*. CO 2 was used throughout the experiment. The pot culture experiment was conducted in the glasshouse, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore and field experiments were carried out at Coconut Research Station (CRS), Aliyar Nagar.

3.1. Isolation of the pathogen

Groundnut leaves, showing typical leaf blight symptoms were collected. Tissues from the edge of the lesions were cut into 0.5 cm bits along with healthy tissues, surface sterilized with 0.1 per cent mercuric chloride, followed by three washings with sterile distilled water. Then, the tissues were placed on potato dextrose agar (PDA) medium. The pathogen was purified by hyphal tip method (Rangaswami, 1972) and maintained on PDA in slants at 4˚C.

3.2. Pathogenicity test

The pathogenicity of the fungus was proved by Koch's postulates, using the susceptible groundnut *cv*. CO 2. The plants were raised in the glasshouse in earthen pots (30 cm dia), filled with pot culture soil mixture. Conidial suspension of the pathogen was prepared according to Sutton and Shane (1983). Conidia were harvested from 20-day-old PDA culture by flooding the plates with sterile distilled water and then scrapping the agar culture three times to obtain sufficient inoculum. The conidial suspension was strained through two layers of cheesecloth and centrifuged at 4000 rpm for 10 min. to remove the nutrient supplied by the medium. Inoculum concentration was determined by using a haemocytometer and adjusted to 10^6 conidia^{-ml} with sterile distilled water. One ml teepol AG, (alkalylbenzene sodium salt) was added to 100 ml conidial suspension to increase the

spreading quality of the suspension. The conidial suspension was sprayed on the leaves of 30-day old groundnut plants maintained in the greenhouse, using an atomizer. Then, all inoculated plants were covered with polythene sheet up to five days to ensure high humidity. Symptom development was observed daily. The pathogen was re-isolated and compared with original isolate. The fungus was identified at Indian Type Culture Collection, IARI, New Delhi.

3.3. Survey for the incidence of *Alternaria alternata*

Survey was conducted during Rabi 2003 in major groundnut growing districts of Tamil Nadu *viz*., Coimbatore, Pudukkottai, Trichy, Thiruvannamalai, Cuddalore, Salem, Vilupuram, Madurai and Dharmapuri for the occurrence of the disease. In each district, one village with four fields were selected randomly. The disease intensity was assessed based on a 1-9 scale (Subrahmanyam *et al*., 1981) as described below.

Score	Disease description	Disease severity (%)
1	No disease	0
2	Lesion present largely on lower leaves, no defoliation	1-5
3	Lesions present largely on lower leaves, very few on middle leaves, defoliation of some leaflets evident on lower leaves.	6-10
4	Lesions present on all lower leaves and the middle leaves but severe on lower leaves; defoliation of some leaflets evident on lower leaves.	11-20
5	Lesions present on all lower leaves and middle leaves, over 50% defoliation on lower leaves	21-30
6	Severe lesions present on all lower leaves and middle leaves, lesions present but less severe on top leaves, extensive defoliation of lower leaves, defoliation of some leaflets evident on middle leaves.	31-40

Score	Disease description	Disease severity (%)
7	Lesion on all leaves but less severe on top leaves, defoliation of all lower and some middle leaves.	41-60
8	Defoliation of all lower and middle leaves, severe lesions on top leaves, some defoliation on top leaves evident	61-80
9	Almost all leaves defoliated, leaving bare stems, some leaflets may remain but show severe leaf spots	81-100

Per cent disease index was calculated by using the following formula.

$$\text{Per cent Disease Index (PDI)} = \frac{\text{Sum of individual rating}}{\text{No. of plants assessed}} \times \frac{100}{\text{Maximum disease category}}$$

3.4. Isolation of biocontrol agents

3.4.1. Isolation of *Pseudomonas* strains from the rhizosphere soil of groundnut

The plants were pulled out gently with roots intact and the excess soil adhering to the roots was removed gently. One gram of rhizosphere soil was transferred to 100 ml of sterile distilled water in a 250 ml Erlenmeyar flask. After thorough shaking, the different strains of *P. fluorescens* were isolated on King's B (KB) medium (King *et al*., 1954) by serial dilution plate method. One ml of bacterial suspension was poured in Petri dish containing King's B medium and incubated at 28 ± 2˚C for 24h. Colonies were viewed under UV light at 366 nm and the same was maintained in (KB) agar slants at 4°C. The Pf1 strain of *P. fluorescens* was obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. The other strains of *P. fluorescens* were isolated from groundnut plants, collected from various places of Tamil Nadu mentioned herein.

Pseudomonas strains isolated from the rhizosphere soil of different districts

Sl. No	***Pseudomonas* strains**	**Location / Districts**
1	*Pseudomonas fluorescens* –Pf2	Alangudi, Pudukkottai
2	*P. fluorescens* –Pf3	Vayalur, Trichy
3	*P. fluorescens* –Pf4	Velavaram, Thiruvannamalai
4	***P. fluorescens* –Pf5**	Valayammadevi, Cuddalore

3.4.2. Isolation of *Bacillus* sp. from the rhizosphere soil of groundnut

Bacillus spp. were isolated from the rhizosphere using nutrient agar (NA) as described earlier. *B. subtilis* strains isolated from the various places were designated as follows.

***Bacillus* strains isolated from the rhizosphere soil of different districts**

Sl. No	***Bacillus* strains**	**Location/ District**
1	*Bacillus subtilis* Bs1	Aliyar, Coimbatore
2	*B. subtilis* Bs2	Alangudi, Pudukkottai
3	*B. subtilis* Bs3	Vayalur, Trichy
4	*B. subtilis* Bs4	**Velavaram, Thiruvannamalai**
5	*B. subtilis* Bs5	Valayamadevi, Cuddalore
6	*B. subtilis* Bs6	Omalur, Salem
7	*B. subtilis* Bs7	Pichanatham, Villupuram
8	*B. subtilis* Bs8	Usilampatti, Madurai
9	*B. subtilis* Bs9	**Thimapuram, Dharmapuri**
10	*B. subtilis* Bs10	**Kalvettupalayam, Erode**

3.4.3. Preparation of bacterial inoculum

King's B and nutrient agar broths were inoculated with *Pseudomonas and Bacillus* strains, respectively. They were shaken constantly for 48 h at room temperature (28 ± 2˚C). Cells were removed by centrifugation at 8000 rpm for 10 min. at 4°C. Finally, an adequate amount of sterile distilled water was added until to obtain bacterial colonies of 10^8 cfu ml^{-1} (Thompson, 1996). In 600 ml of bacterial inoculum, 1 kg talc powder and 10 g carboxy methyl cellulose (CMC) was added. The groundnut seed was treated with the bacterial formulation at the rate of 10g kg^{-1} of seed.

3.5. *In vitro* screening of *Pseudomonas fluorescens* on radial mycelial growth of *A. alternata*

Pseudomonas fluorescens strains *viz*., Pf1, Pf2, Pf3, Pf4 and Pf5 were screened against mycelial growth of *A. alternata* by dual culture technique (Dennis and Webster, 1971). The bacterial culture was streaked on one side of the Petri dish (1 cm from the edge of the plate) containing PDA medium and mycelial disc (9 mm) of seven day-old culture of *A. alternata* was placed on the opposite side of the bacterial streak. Three replications were maintained for each treatment, in a randomized completed block design. The plates were incubated at room temperature (28 $\pm$ 2°C) and the zone of inhibition was measured. The mycelial growth of the pathogen and per cent reduction over control was calculated by using the formula.

$$\text{Per cent reduction over control} = \frac{\mathbf{C - T}}{\mathbf{C}} \times 100$$

where,

C – mycelial growth of pathogen in control

T – mycelial growth of pathogen in dual plate

3.6. *In vitro* screening of *B. subtilis* strains on radial mycelial growth of *A. alternata*

The *Bacillus* strains *viz*., Bs1, Bs2, Bs3, Bs4, Bs5, Bs6, Bs7, Bs8, Bs9 and Bs10 were screened against radial mycelial growth of *A. alternata* in dual culture technique, as described earlier.

3.7. *In vitro* screening of plant extracts against *A. alternata*

3.7.1. Preparation of Plant extracts

The presence of antifungal principles (AFPs) in leaf extracts of various plant species were tested. The extracts were prepared by grinding the leaves in a mortar-using pestle by adding water at the rate of 10 ml g^{-1} of plant tissue and filtered through thin layer of cotton wool. The plant extract was diluted further to have 10 per cent concentration (v/v).

3.7.2. *In vitro* evaluation of plant extracts against *A. alternata*

Leaf extracts of following plant species were tested for their efficacy against *A. alternata.*

Sl. No	Scientific name	Common/Vernacular name	Family
17.	*Abutilon indicum L.*	Thuthi	Malvaceae
18.	*Azadirachta indica L.*	Neem	Meliaceae
19.	*Bougainvillea spectablis L.*	**Bougainvillea**	Nyctaginaceae
20.	*Catharanthus roseus L*	Periwinkle	Apocynaceae
21.	*Acalypha indica*	Kuppaimeni	Acanthaceae
22.	*Ocimum sanctum*	Tulsi	Labiatae
23.	*Lawsonia inermis*	Maruthani	Sonneratiaceae
24.	*Datura metel*	Umathai	Solanaceae
25.	*Prosopis juliflora*	Velikaruvel	Mimoceae
26.	*Tridox procumbens*	Vettukayapoondu	Compositae

Sl. No	Scientific name	Common/Vernacular name	Family
27.	*Cyprus rotandus*	**Koraipul**	Cyperaceae
28.	*Vitex negundo*	Notchi	Verbanaceae
29.	*Ipomea cornea*	Velikattamanakku	Convolvulaceae
30.	*Cynadon dactylon*	Haryali	Graminae
31.	*Pongamia glabra*	Pungam	Leguminaceae
32.	*Neerium indicum*	Arali	Apocynaceae

Poisoned food technique (Schmitz, 1930) was followed to study the antifungal activity of various plant extracts. Ten ml of respective plant extract (100 %) was mixed with 90 ml of PDA to obtain 10 per cent concentration and sterilized at 15 lbs pressure for 30 min. Twenty ml of amended medium was poured into sterile Petri plates and 9 mm mycelial disc of the test fungus *A. alternata* was placed on the centre of the plate. They were incubated at room temperature (28 ± 2^{o}C). Three replications were maintained for each treatment in a randomized complete block design. PDA without leaf extract having sterile distilled water served as control. Per cent reduction of mycelial growth over control was calculated.

$$\text{Per cent reduction over control} = \frac{C - T}{C} \times 100$$

where,

C – mycelial growth of pathogen in control

T – mycelial growth of pathogen in poisoned media

3.8. Plant growth promotion by biocontrol agents

The plant growth promoting activity of biocontrol agents was assessed based on seedling vigour index (ISTA, 1966). The germination test was conducted in sand

medium, using 50 groundnut seeds taken at random. Eight replications were maintained in a randomized complete block design. The temperature of the germination chamber was maintained at 25 ± 2^{o} C with RH of 90 ± 3 per cent. Per cent of seed germination, root length and shoot length of individual seedlings were measured. The vigour index was calculated by using the formula described by Abdul Bagi and Anderson (1975).

Vigour index = (Mean root length + Mean shoot length) x Germination (%)

3.9. Bacterial metabolites

3.9.1. Salicylic acid production

Salicylic acid production of the Pf strains was determined as per the method of Meyer *et al*. (1992). The bacterial strains were grown in the standard succinate medium (Succinic acid- 4.0g, K_2HPO_4-6.0g, KH_2PO_4 3.0g, $(NH_4)_2SO_4$ 1.0g, $MgSO_4$ $7H_2O$-0.2g, distilled water-1000ml, pH 7.0) at 28 ± 2°C for 48 h. Cells were collected by centrifugation at 6000 rpm for 5min. Four ml of cell free culture filtrate was acidified with 1N HCl to pH 2.0 and salicylic acid was extracted with equal volume of chloroform. Four ml of water and 5µl of 2M $FeCl_3$ were added to the pooled chloroform phases. The absorbance of the purple iron salicylic acid complex, which was developed in the aqueous phase was read at 527nm. Salicylic acid was measured according to a standard curve drawn with salicylic acid dissolved in succinate medium and treated as described above. The quantity of salicylic acid was expressed as μgml^{-1} of the culture filtrate.

3.9.2. Siderophore production

The strains of *P. fluorescens* were grown in KB broth for 3 days and centrifuged at 2000 rpm for 10 min. The pH of supernatant was adjusted to 2.0 with HCl and equal quantity of ethyl acetate was added into separating funnel, mixed well and ethyl acetate fraction was collected. This was repeated three times to bring the entire quantity of

siderophore from the supernatant. The ethyl acetate fractions were pooled, air-dried and dissolved in 5 ml of ethanol (50%). Five ml of ethyl acetate fraction was reacted with 5 ml of Hathway's reagent (1.0 ml of 0.1 M $FeCl_3$ in 0.1 N HCl to 100 ml of distilled water + 1.0 ml of potassium ferriccyanide). The absorbance of dihydroxy phenol was read at 700 nm. A standard curve was prepared using dihydroxy benzoic acid (Dileep *et al*., 1998). The quantity of siderophore synthesized was expressed as μg ml^{-1} of culture filtrate.

3.9.3. Indole Acetic Acid (IAA) production

Production of indole acetic acid (IAA) by *P. fluorescens* in the medium was assayed by the method described by Gorden and Paleg (1957). The *P. fluorescens* was grown in triptic soy broth (TSB) (animal peptone 15.0 g; Soya peptone 5.0 g; NaCl 5.0g; glycine 4.4g; distilled water 1000 ml) with tryptophan as a precursor (100 μg / ml) and incubated in a rotary shaker for 30 h. Supernatant from the cultures were collected after centrifugation at 2000 rpm for 10 min. To one ml of cell free culture filtrate, 2 ml Salkowsky reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HclO_4$) was added and incubated at 28 ± 2°C for 30 min. The absorbance was measured at 530 nm. A standard curve was prepared with IAA and quantity of IAA produced was expressed as μg ml^{-1}.

3.9.4. Hydrogen cyanide (HCN) production

3.9.4.1. Qualitative assay

Production of HCN was determined by the method described by Miller and Higgins (1970). Bacteria were grown on tryptic soy agar (TSA). A sterile filter paper disc of 1.5cm was soaked in picric acid solution (2.5 g of picric acid, 12.5 g of Na_2 CO_3, 1000 ml of distilled water) and placed in the upper lid of each Petri dish. Dishes were sealed with parafilm and incubated at 28^{o}C for four days. Hydrogen cyanide production was

assessed by the change of light yellow colour of filter paper to brown or reddish brown as an indication of weak, moderate or strong production of HCN by each strain.

3.9.4.2. Quantitative assay

Bacteria were cultured on tryptic soy broth (TSB) in sterile conical flasks. Filter paper was cut into uniform strips of 10 cm length and 0.5 cm wide, saturated with alkaline picrate solution and a single filter paper was placed inside the each conical flask in a hanging position. After incubation at 28 ± 2°C for 48 h, the sodium picrate in the filter paper was reduced to reddish compound in proportion to the amount of hydrocyanic acid evolved. The filter paper was placed in a clean test tube containing 10 ml of distilled water and the colour was eluted and absorbance was measured at 625 nm (Sadasivam and Manickam, 1992).

3.10. Estimation of biochemical constituents of plant products

3.10.1. Phenolic content

The phenolic content of leaf samples *viz., A. indicum, D. metel, L. inermis* and *A. indica* was determined as per the procedure given by Zieslin and Ben-Zaken (1993). One gram of the tissue was homogenized in 10 ml of methanol (80%) and agitated for 15 min. at 70^{o}C. One ml of the methanolic extract after evaporation was added to 5 ml of distilled water and 250 ml of Folin Ciocalteau reagent (1 N) was added and the solution was kept at 25^{o}C. After three min, one ml of saturated solution of $Na_2 CO_3$ and one ml of distilled water was added and the reaction mixture was incubated for 1h at 25^{o}C. The absorbance of the developed blue colour was measured using a Hitachi 200-20 Spectrophotometer at 725 nm. The content of total soluble phenols was calculated according to a standard curve obtained from a Folin – Ciocalteau reaction with a phenol solution (C_6H_6O) and expressed as phenol equivalents in mg g^{-1} fresh tissue.

3.10.2. Protein content

Protein was estimated by the method of Bradford (1976). Ten mg of Coomassie brilliant blue G-250 was dissolved in 4.7 ml of absolute alcohol and 10 ml of concentrated phosphoric acid and the volume was made up to 100 ml with distilled water. A sample of 50 µl was added to 950 µl of dye solution and the mixture was incubated for 5 min at room temperature. The absorbance was recorded at 595 nm in Hitachi 200-20 Spectrophotometer. Bovine serum albumin was used as standard.

3.10.3. Purification of proteins

Ten gram of leaf sample was ground with 20 ml of sodium phosphate buffer (pH 7.0), filtered and centrifuged at 10,000 rpm for 20 min at 4^{o}C. The supernatant was then precipitated with 80 per cent saturated ammonium sulphate and a pellet was obtained after centrifugation further at 12,000 rpm for 20 min. The pellet was resuspended in minimal amount of distilled water and finally dialyzed against 70 mM potassium phosphate buffer, pH 6.0.

3.11. Thin Layer Chromatography (TLC)

3.11.1. Preparation and activation of TLC plates

Silica Gel-G (E-Merck) was coated on TLC (20x20 cm) plate to form thin layer. Twenty gram of fine silica gel-G powder was mixed thoroughly with 40 ml of distilled water and applied to TLC plate by using applicator, to form 0.5 mm thickness. A fine layer of 0.5 mm thick wet silica gel was formed on the glass plate. The glass plate was allowed to dry in open air for 1 h and then in hot air oven at 110^{o}C for 2 h. The activated TLC plate was applied with leaf samples (20 µl) with the help of micropipette.

3.11.2. Separation of compounds by TLC

The solvent system used was chloroform : methanol (90:10) for phenolic compounds and (15:5) for alkaloids. The TLC plate was slowly placed into the tank so as to immerse 0.5 cm into the solvent at the bottom. The tank was closed with a glass lid so as to have the chamber completely filled with the solvent vapours. When the solvent front reached the end of the plate, the plate was removed from the tank and kept in open air at room temperature to evaporate the solvent.

3.11.3. Identification of compounds

The dried TLC plate was sprayed and exposed to various reagents so as to identify the compounds present in the leaf extract.

3.11.3.1. Spray reagents

3.11.3.1.1 Dragendorff's reagent

Dragendorff's reagent was used to identify the presence of alkaloids.

Solution A: 1.7 g bismuth nitrate was dissolved in 100 ml of water: acetic acid (8:2)
Solution B: 40 g of potassium iodide was dissolved in 100 ml water.
Solution C: 20 ml of acetic acid was mixed with 70 ml water.

Finally 5 ml of A and 5 ml of B were added to the solution C to make up to 100 ml just before use. Dried plate was sprayed with the above solution. Presence of red, orange, brown and yellow spots indicated the presence of alkaloids.

3.11.3.1.2. Diazotized sulphanilic acid solution

Fifty mg of diazotized sulphanilic acid was added to 20 ml of 20 per cent Na_2CO_3 solution and sprayed on the dried TLC plates. Yellow, orange and red coloration indicated the presence of phenols.

Diazotized sulphanilic acid was prepared by dissolving 25 g of sulphanilic acid in 125 ml sodium nitrate (10 %) solution. This mixture was added drop by drop to 60 ml 8 M HCl under ice-cold condition. After 10 min, this mixture was filtered in cold water and then washed with ethanol and ether repeatedly. The crystals were air dried and stored at 4^{o}C.

3.11.3.1.3. Folin-Ciocalteau reagent

This reagent was used for identification of phenols. Folin- Ciocalteau reagent was diluted (1:1) with water and sprayed on the dried TLC plate followed by 20 per cent Na_2CO_3 solution. Presence of blue spots indicated the presence of phenolic compounds.

3.11.4. Isolation of TLC separated compounds

The TLC technique was also used for isolation of separated compounds and in that case it is called as preparative TLC. Instead of thin layer, a thick layer (2mm) of absorbent (silica gel) was coated and a greater quantity of the sample was applied onto the plate as a streak rather than a spot. After separation, the area of the separated compound was scraped off, eluted with methanol and recovered in a relatively pure form.

3.11.5. Antifungal activity of separated compounds

To test the antifungal activity of the separated compounds, PDA medium was melted, cooled and then 10 ml of the medium was poured into the sterilized Petri plates and allowed to solidify. Three wells were made in solidified media near the periphery of the plate. Later nine mm mycelial disc of *A. alternata* was kept in the middle of the Petri dish. The wells were filled with the partially purified bioactive compounds and incubated at room temperature (28 ± 2^{o} C) for eight days. After incubation period, the plates were examined for the inhibition of the mycelial growth.

3.12. Induction of defense mechanism against *A. alternata*

The promising biocontrol agents *viz*., Pf1, Pf2, Bs1, Bs 5, and plant products of *A. indicum* and *D. metel* effective under pot culture condition were selected for testing their activity for the induction of defense mechanism. The experiment consisted of the following treatments.

Treatment details

T1	ST+FS (Pf1)
T2	ST+FS (Pf1+PI)
T3	ST+FS (Pf2)
T4	ST+FS (Pf2+PI)
T5	ST+FS (Bs1)
T6	ST+FS (Bs1+PI)
T7	ST+FS (Bs5)
T8	ST+FS (Bs5+PI)
T9	*A. indicum* (10 %)
T10	*A. indicum*+PI
T11	*D. metel* (10 %)
T12	*D. metel* + PI
T13	Inoculated control
T14	Healthy

ST – Seed treatment – 10g/kg, FS - Foliar spray - 0.2%, PI-Pathogen inoculation

The seeds were treated with talc based formulation of biocontrol agents @ 10 g kg^{-1} of seed. Thirty days after sowing, the plants were sprayed with the biocontrol agents @ 0.2 per cent concentration and leaf extracts @ 10 per cent concentration

separately. The plants were challenge inoculated with the pathogen one-day after the spray. Plants inoculated with the pathogen and uninoculated healthy were also maintained. In each treatment three replications were maintained in a randomized complete block design.

The groundnut leaves were collected from the treated plants at different intervals *viz*., 1, 3, 5 and 7 days after challenge inoculation with the pathogen and used for the analysis of defense related enzymes.

One gram of leaf tissue was homogenized with 1 ml of sodium phosphate buffer (pH 7.0). Homogenized samples were centrifuged at 10,000 rpm for 10 min. The supernatant solution was used as source for analyzing phenylalanine ammonia-lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), β-1, 3 glucanase, chitinase, tyrosine ammonia lyase (TAL), protein and phenol.

3.12.1. Assay of Phenylalanine Ammonia Lyase (PAL) activity

Leaf samples (1 g) were homogenized in 3 ml of ice cold 0.1 M sodium borate buffer, pH 7.0 containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinyl pyrrolidone. The extract was filtered through cheese cloth and the filtrate was centrifuged at 16,000 rpm for 15 min. The supernatant was used as enzyme source. Phenylalanine ammonia lyase activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm as described by Dickerson *et al.* (1984). Sample containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 0.l M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30^{o}C. The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient of 9630 M^{-1}. Enzyme activity was expressed as nmol trans-cinnamic acid min^{-1} mg^{-1} of protein.

3.12.2. Assay of Peroxidase activity (PO)

One gram of fresh leaf tissue was ground with 2 ml of 0.1M phosphate buffer, pH 7.0 at 4^{o}C. The homogenate was centrifuged at 6000 g at 4^{o} C for 10 min. and the supernatant was used as enzyme source. The reaction mixture consisted of 1.5 ml of 0.05M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of one per cent H_2O_2. The reaction mixture was incubated at room temperature (28 ± 2°C). The change in absorbance was recorded at 420 nm at 30 sec interval for 3 min. The boiled enzyme preparation served as blank. The enzyme activity was expressed as changes in the absorbance of the reaction mixture min^{-1} mg^{-1} of protein (Hammerschmidt *et al.,* 1982).

3.12.3. Assay of Polyphenol oxidase activity (PPO)

Polyphenol oxidase activity was determined as per the procedure given by Mayer *et al.* (1965). One gram of leaf sample was homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 16,000 rpm for 10 min at 4°C and the supernatant was used as the enzyme source. The reaction mixture consisted of 200 µl of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and to start the reaction, 200 µl of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm min^{-1} mg^{-1} of protein.

3.12.4. Assay of Chitinase

The colorimetric assay of chitinase was carried out according to the procedure developed by Boller and Mauch (1988).

a. Preparation of colloidal chitin

Colloidal chitin was prepared by treating 1 g of crab-shell chitin powder with acetone to form a paste and 20 ml of concentrated hydrochloric acid (HCl) was added slowly while grinding in a mortar with the temperature maintained at 5^{o}C. After several minutes the content was then filtered through glass wool and collected in ice cold ethanol by constant stirring to precipitate the chitin in a highly dispersed state. The residue was

sedimented and resuspended in distilled water several times to remove excess acid and alcohol and then dialyzed against tap water. Chitin content of the suspension was determined by drying a sample *in vacuo* and adjusted with distilled water to a final concentration of 10 mg ml^{-1} (dry weight/ volume) and stored at $5^{o}C$ (Berger and Reynolds, 1958).

b. Snail gut enzyme

Six hundred mg of the commercial lyophilized snail gut enzyme (Helicase, obtained from Sepracor, France) was dissolved in 10 ml of 20 mM potassium chloride (KCl) and chromatographed on a Sephadex G-25 column (38 x 1.5 cm) using a 10 mM KCl solution, containing 1mM EDTA and adjusted to pH 6.8 for equilibration and elution. The first 20 ml was eluted after the void volume collected (Boller and Mauch, 1988).

c. Preparation of p-dimethylaminobenzaldehyde (DMAB) reagent

The DMAB reagent was prepared by the procedure described by Reissig *et al.* (1959). Stock solution of DMAB was prepared by mixing 8g of DMAB in 70 ml of glacial acetic acid along with 20 ml of concentrated HCl. One volume of stock solution was mixed with 9 volumes of glacial acetic acid immediately before use.

d. Enzyme extraction

One gram of groundnut leaves were extracted with 5 ml of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 10,000 rpm and the supernatant was used as an enzyme source.

e. Assay procedure

The assay mixture consisted of 10 µl of 1 M sodium acetate buffer (pH 4.0), 0.4 ml of enzyme extract and 0.1 ml of colloidal chitin (0.1%). After 2 h of incubation at 37^{o}C, the reaction was stopped by centrifugation at 1000 g for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted out and transferred into a glass reagent tube containing 30 ml of 1 M potassium phosphate buffer (pH 7.1) and incubated with 20 µl of (w/v) desalted snail gut enzyme (3%) (w/v) for 1 h. After 1 h, the pH of the reaction mixture was brought to 8.9 by the addition of 70 µl of 1 ml sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min and then rapidly cooled in an ice water bath. After addition of 2 ml of DMAB reagent, the mixture was incubated for 20 min at 37^{o}C. Immediately thereafter, the absorbance value at 585 nm was measured using a Hitachi 200-20 Spectrophotometer. N-acetyl glucosamine (GlcNAc) was used as standard (Boller and Mauch, 1988). The enzyme activity was expressed as nmol GlcNAc min^{-1} mg^{-1} fresh tissue.

3.12.5. Assay of β-1, 3- glucanase

β-1, 3- glucanase was analyzed by the laminarin-dinitro salicylic acid method (Pan *et al*., 1991). The reaction mixture consisted of crude enzyme extract of 62.5 µl and 62.5 µl of 4% laminarin. The reaction was carried out at 4° C for 10 min. and the reaction was stopped by adding 375 µl of dinitro salicylic acid and heated for 5 min. on a boiling water bath. The resulting solution was diluted with 4.5 ml distilled water and the absorbance was read at 500 nm. The crude extract preparation with laminarin at zero time incubation served as blank. The enzyme activity was expressed as µg equivalent of glucose released min^{-1} mg^{-1} of protein.

3.12.6. Assay of Tyrosine Ammonia Lyase activity (TAL)

One gram of leaf sample was homogenized in 3 ml of 0.1 M sodium borate buffer (pH 7.0) containing 30 µg insoluble polyvinyl pyrolidone (PVP). The homogenate was filtered through four layers of cheese cloth. The filtrate was centrifuged at 20,000 g for 30 min at 4^{o}C. The supernatant served as the source of enzyme extract. The reaction mixture consisted of 50 µl enzyme extract, 2.45 ml of 1M sodium borate buffer (pH 8.8) and 0.5 ml of 2mM tyrosine. The absorbance values were read spectrophotometrically at 290 nm. The TAL activity was expressed as change in absorbance (in units) min^{-1} g^{-1} fresh tissue (Dickerson *et al*., 1984).

3.12.7. Phenolic content

The phenolic content of the groundnut leaf sample was determined as per the procedure given in the section 3.10.1.

3.12.8. Protein

Protein content of the groundnut leaf sample was determined by Bradford's method as given in the section 3.10.2.

3.13. Native polyacrylamide gel electrophoretic analysis (Native PAGE)

The isoform profile of PO and PPO were examined by discontinuous native poly acrylamide gel electrophoresis analysis (Native PAGE) (Laemmli, 1970). Leaf samples showing maximum activity of PO and PPO in spectrophotometer assay were used for analysis. Induction of defense reaction against *A. alternata* in groundnut was tested for the activity of PO and PPO and was found to be maximum on 5th day after challenge inoculation. Hence leaf samples collected on 5th day after challenge inoculation with *A. alternata* were used for the isozyme analysis.

3.13.1. Isozymes

Seed treatment and foliar spray with biocontrol formulation and plant products were done as described earlier. One set of 30 days old plants treated with the biocontrol agents Pf1, Pf2, Bs1 and Bs5 while another set of treated plants were challenge inoculated with the pathogen. Healthy and inoculated plants were also maintained. After five days of inoculation with the pathogen, samples were collected and analyzed for the induction of isozyme.

3.13.1.1. Peroxidase isozyme (PO)

Groundnut leaf samples were homogenized in 0.1 M sodium phosphate buffer pH 6.0 (2 ml/g fresh tissue) (Smith and Hammerschmidt, 1988). Peroxidase isozymes were analyzed as per the procedure given by Pan *et al.* (1991) by native polyacrylamide gel electrophoresis (PAGE). The gels were run for 8 h at 10^{o}C (constant current: 30 mA) with a Hoefer vertical electrophoresis unit. Samples of 100 µl containing 1 µg protein were electrophoresed at 30 mA for 6 h. Gel was stained for peroxidase with 0.2 M acetate buffer at pH 4.5 containing benzidine (0.05%) and H_2O_2 (0.03%). After staining, the gel was immersed in acetic acid (7%) for 3 min and washed with distilled water (Nadlony and Sequeira, 1980).

3.13.1.2. Polyphenol oxidase isozymes (PPO)

The PPO was assayed by homogenizing 1 g of tissue in 0.01 M potassium phosphate buffer (pH 7.0) containing non-ionic detergent (Tween 20) (1%) at 4^{o}C for 15 minutes. The homogenate was centrifuged at 20,000 g for 15 min at 4^{o}C in a refrigerated centrifuge and the supernatant was used as the enzyme source. After native PAGE, the gel was equilibrated for 30 min in p-phenylene diamine (0.1%) in 0.1 M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer.

The addition of catechol was followed by a gentle shaking which resulted in appearance of dark brown discrete protein bands (Jayaraman *et al.,* 1987).

3.14. Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

One gram of leaf sample was ground in a chilled pestle and mortar with 2 ml of ice-cold potassium phosphate buffer (0.1M) at pH 6.5. The buffer extracts were then centrifuged at 10,000 rpm for 20 min. at 4^{o}C and the supernatant was used for the SDS-PAGE. The protein content of the sample was determined by the procedure as described by Bradford (1976).

Hundred µl of protein from treatments were taken and mixed with 10 µl of sample buffer in microfuge tube, boiled for 4 min. and incubated at 4^{o} C for 30 min. Then the samples containing equal amount of proteins were loaded in to the wells of polyacrylamide gels (Annexure) (Sigma Aldrich Techware System, Sigma, USA). The medium molecular weight markers (GENEI, Bangalore) were used. Electrophoresis was carried out at constant voltage of 75V. The gels were stained with Coomassie brilliant blue (G 250) solution based on the Rf value of each protein band stained, the molecular weight was calculated.

3.15. Screening of biocontrol agents and plant products against *A. alternata* under green house condition

The promising biocontrol agents (Pf1, Pf2, Bs1 and Bs5) and the plant products (*A. indicum* and *D. metel*) under *in vitro* condition, were further tested against *A. alternata* under greenhouse condition. The treatments were given as seed treatment (ST), seed treatment plus foliar application (ST+FS) and foliar spray (FS) alone as described below.

Treatment details

T1	Pf1 (ST)
T2	Pf1 (ST+FS)
T3	Pf1 (FS)
T4	Pf2 (ST)
T5	Pf2 (ST+FS)
T6	Pf2 (FS)
T7	Bs1 (ST)
T8	Bs1 (ST+FS)
T9	Bs1 (FS)
T10	Bs5 (ST)
T11	Bs5 (ST+FS)
T12	Bs5 (FS)
T13	*A. indicum* (10%) (FS)
T14	*D. metel* (10%) (FS)
T15	Mancozeb 75 % WP (0.2%)- (FS)
T16	Untreated control

ST - Seed treatment - 10g /kg, FS-Foliar spray - 0.2 per cent

Foliar sprayings were given at fortnightly intervals, starting from 30 days after sowing. The observation on disease intensity was recorded at 90 DAS on a 1-9 scale as described earlier. Various parameters like plant height and number of branches were also recorded at 30, 45 and 70 DAS. At the time of harvest, yield and biomass production were recorded.

3.16. Efficacy of promising biocontrol agents and botanicals against groundnut leaf blight disease (*A. alternata*), under field conditions

Field experiment were conducted at CRS, Aliyar, to evaluate the promising (greenhouse) biocontrol agents of Pf1, Pf2, Bs1, Bs 5 and plant products *viz., A. indicum* and *D. metel* against *A. alternata.* The field trials were laid out with eight treatments replicated thrice in a randomized complete block design, using a plot size of 4x3m. The observations on disease intensity was recorded at 90 DAS, on a 1-9 scale as described earlier. The yield and total biomass production were recorded during harvest.

Treatment details

T1	Pf1 (ST+FS)
T2	Pf2 (ST+FS)
T3	Bs1 (ST+FS)
T4	Bs5 (ST+FS)
T5	*A. indicum* 10%
T6	*D. metel* 10%
T7	Mancozeb 75 % WP (0.2%) (FS)
T8	Untreated control

ST- Seed treatment at 10 g / kg.

FS- Two foliar sprays at fortnightly intervals starting from 30 DAS.

3.17. Detection of *A. alternata* by Enzyme Linked Immunosorbent Assay (ELISA)

3.17.1. Raising of Polyclonal antibodies

A. alternata was grown on PDA broth for 15 days. Freshly harvested mycelial mat was ground in 0.1 M phosphate buffer (pH 7.0) and clarified at 12,000 rpm for 10 min at room temperature. One ml of the antigen was mixed with one ml of Freund's complete adjuvant, saline and emulsified in a cyclomixer. The emulsion was given intramuscularly (4 months old New Zealand white rabbits weighing about

2.5 kg) with sterile syringe for production of PAbs (polyclonal antibodies) against *A. alternata* mycelial protein, after adjusting the protein content to 150 g ml^{-1} (Bradford, 1976). Four injections were given at weekly intervals however, the fourth injection was an incomplete adjuvant. Bleeding was done 15 days after the last injection and the blood was transferred to sterile glass vials and allowed to stand in a slanting position until coagulated. The antiserum was transferred to sterile centrifuge tubes and the red blood cells were pelleted by centrifugation (10,000 rpm, 4°C, 10 min) three times. The antiserum was stored at 4°C for further studies.

3.17.2. Antigen preparation

Leaf samples were collected from both healthy and infected plants. After washing, the samples were ground in 0.1 M Carbonate buffer (pH 9.6) in a sterile pestle and mortar at room temperature (28 $\pm$ 2°C) and clarified at 12,000 rpm for 10 min. at 4°C. The supernatant was stored at 4°C for further studies.

3.17.3. Direct Antigent Coating (DAC) ELISA

DAC-ELISA method (Clark and Adams, 1977) was used for detection of *A. alternata*. Microtitre plates (Tarson, India) were first coated with 100 µl of antigen for 2 h at 37°C. The plates were washed three times with PBS-T. The primary antibodies diluted in PBS-T (1:1000) containing two per cent polyvinyl pyrrolidone and 0.2 per cent ovalbumin (PBS-TPO) were added (100 µl per well) separately. After incubation at 37°C (2 h), the plates were washed using PBS-T. Alkaline phosphatase (ALP) conjugated goat anti-rabbit immunoglobulin (Bangalore Genei, India) (1:6000 with PBS-TPO) were added @ 100 µl per well. The plates were incubated for 2 h at 37°C. After washing in PBS-T, 100 µl of 4-nitrophenyl phosphate (SD Fine chemicals, India) dissolved in diethanolamine at 1 mg ml^{-1} (Sigma, USA) (pH 9.8) was added. The colour development (absorbance) was read at 405

nm with a microplate reader (Bio Rad Model 3550, USA). The reaction was terminated by adding 50 µl of 3 M NaOH after incubation for half an hour at room temperature (28 $\pm$ 2°C).

3.18. Studies on the role of weather parameters on the incidence of *A. alternata*

Three field trials were conducted to find out the effect of various weather parameters on disease incidence, during Rabi 2002-'03, Kharif 2003-'04 and Rabi 2003-'04 at CRS, Aliyar Nagar. For each season, three sowings were taken at fortnightly intervals with two treatments *viz.*, insect pest control and control plot without any treatment. The insect pest control treatment was maintained to keep the plants free from insect pest infestation. A plot size of 120 square metres was adopted for each treatment. The susceptible groundnut *cv*. CO 2 was sown with a spacing of 30x10 cm. Disease intensity was assessed at weekly intervals, after onset of the disease. For observation, ten plants were selected randomly, tagged and in each plant 3 trifoliate leaves *viz*., upper, middle and lower leaves were selected. The disease intensity was recorded based on 1-9 scale as described earlier.

Various direct weather parameters *viz*., maximum and minimum temperature, relative humidity, rainfall and bright sunshine hours and the derived weather parameters *viz*., Growing Degree Days (GDD), Diurnal Variation (DV) and Relative Temperature Disparity (RTD) were recorded and correlated with disease intensity.

where,

$$\mathbf{DV = Maximum\ temperature - Minimum\ temperature\ of\ a\ day.}$$

$$RTD = \frac{\text{Maximum temperature - Minimum temperature}}{\text{Maximum temperature}} \times 100$$

$$GDD = \frac{\left(\text{Maximum temperature} + \text{Minimum temperature} \right)}{2} - \text{Base temperature}$$

where,

Base temperature for groundnut = $18^{o}C$

For correlation and regression analysis, the average weather parameters that prevailed before 10 days of observation were recorded based on the time taken for symptom development. The correlation and multiple regression analysis were done by using MSTAT software.

3.19. Statistical analysis

The statistical analysis of the data was made using package developed by IRRISTAT version 92 of International Rice Research Institute Biometrics Unit, The Philippines.

CHAPTER IV

RESULTS

4.1. Pathogenicity and Symptomatology

The pathogenicity of the fungus was proved on 30-day-old groundnut susceptible *cv*. Co.2. Typical symptoms similar to that of naturally infected plants were exhibited within ten days of inoculation. The symptoms were initially characterized by the appearance of yellowing symptoms at the apical portion of the leaves and advanced towards the midrib of the leaves. The chlorotic lesion turned to light to dark brown and became brittle. The fungus was identified as *Alternaria alternata* (Fr.) Keissler at Indian Type Culture Collection (ITCC) of Indian Agricultural Research Institute (IARI), New Delhi (Plate 1 and 2).

4.2. Occurrence of Alternaria leaf blight in major groundnut growing areas of Tamil Nadu

An intencive survey was conducted in ten major groundnut growing districts of Tamil Nadu during Rabi 2003, to assess the disease intensity of Alternaria leaf blight. The disease incidence was maximum in Cuddalore district (63.22 PDI), followed by Salem (62.27 PDI), Coimbatore (60.12 PDI), Madurai (59.35 PDI), Pudukkottai (57.83 PDI), Viluppuram (51.33 PDI), Erode (46.66 PDI), Dharmapuri (42.55 PDI) and Thiruvannamalai (38.33PDI). Minimum disease incidence was observed in Trichy district with 33.77 per cent disease index (Table 1).

4.3. Efficacy of bacterial strains and plant products against of *Alternaria alternata*

4.3.1. *In vitro* screening of *P. fluorescens* strains against *A. alternata*

Among the five strains, Pf1 significantly recorded lowest radial mycelial growth of *A. alternata* **(**43.1 mm). This was followed by Pf2 (48.1 mm) and Pf3

(47.2 mm) compared to control (88.83 mm). However, two strains of *P. fluorescens*, Pf1 and Pf2 were significantly superior in recording higher inhibition zone of 16.20 and 15.30 mm, respectively (Table 2; Plate 3).

4.3.2. *In vitro* screening of *Bacillus subtilis* strains against *A. alternata*

Among the ten strains of *B. subtilis*, Bs1 recorded lesser radial mycelial growth of *A. alternata* **(**41.30 mm), followed by Bs5 (43.30 mm). The strain Bs1 was significantly superior followed by BS 5 by recording maximum higher inhibition zone of 21.00 mm and 19.30 mm (Table 3; Plate 4).

4.4. Effect of leaf extracts against *A. alternata*

Among the sixteen plant species, all the leaf extracts at 10 per cent concentration were effective in inhibiting the radial mycelial growth of *A. alternata*, compared to control. However, *Abutilon indicum* was significantly superior than others by recording the least mycelial growth of 21.20 mm, followed by *Datura metel* (25.20 mm), compared to control (86.50 mm) (Table 4; Plate 5).

4.5. Effect of selective *Pseudomonas* and *Bacillus* strains on plant growth promotion

The promising strains under *in vitro* conditions were evaluated for the presence of growth promoting substances. The results revealed that all the four strains were recorded the presence of growth promoting substances, compared to control. Among these strains, Pf1 was significantly superior than others by recording higher germination percentage of 76.00, followed by 70.70, 70.70 and 66.00 per cent, respectively in Pf2, Bs1 and Bs5, as against 56.00 per cent in control. In case of root length, Pf1 recorded highest root length of 11.30 cm followed by 10.50, 10.30 and 8.40 in Pf2, Bs1 and Bs5, respectively compared to

7.40 cm in control. Pf1 and Pf2 were also recorded highest shoot length of

17.90 cm, compared to control (14.60 cm). The seedling vigour index was more in Pf1 (2212.92) as against 1232.00 in control (Table 5; Plate 6).

4.6. Bacterial metabolites production

4.6.1 Indole acetic acid (IAA) production by *P. fluorescens*

Among the strains of *P. fluorescens*, Pf1 showed the maximum IAA production of 54.70 µg ml^{-1}, followed by Pf2, Pf 3, Pf4 and Pf 5 with 48.30, 34.70, 34.51 and 30.10 µg $ml^{-}1$, respectively (Table 6).

4.6.2. Salicylic acid (SA) production by *P. fluorescens*

Among the different strains, maximum production of salicylic acid (28.10 µg ml^{-1}) was recorded in Pf1 followed by Pf2 (26.80), Pf 3 (15.40), Pf 4 (13.50) and Pf5 (13.12) (Table 6).

4.6.3. Siderophore production by *P.fluorescens*

In case of siderophore production, Pf1 and Pf 2 were equally effective by recording 9.30 and 8.80 micromoles benzoic acid ml^{-1} respectively. This was followed by Pf3 (5.80), Pf4 (5.50) and Pf5 (5.38) (Table 6).

4.7. Hydrogen cyanide (HCN) production by *P. fluorescens* strains

All the Pf1 strains showed high intensity of HCN production. In the quantitative production of HCN, Pf1 and Pf2 significantly produced more amount of HCN with 0.080 and 0.070 (1 unit = 0.001) absorbance, respectively. Other strains *viz*., Pf3 and Pf4 recorded 0.070, followed by Pf5 with 0.050 (Table 7; Plate 7).

4.8. Identification of antifungal chemicals in the plant extracts

4.8.1 Total phenol and protein content of leaf extracts

The maximum total phenol content was observed in *A. indicum* with 242.47 µg of catechol g^{-1} of fresh tissue, followed by *D. metel, L. inermis* and *A. indica* which had 234.39, 184.21 and 173.41 µg g^{-1} of phenols respectively.

The maximum amount of soluble protein content was observed in *A. indicum* (1.421) followed by *D. metel, L .inermis* and *A. indica* with 1.337, 1.233 and 1.131 mg g^{-1}, respectively (Table 8).

4.8.2. Detection of active substances by Thin Layer Chromatography

The results revealed that the spots with Rf values of 0.745, 0.518, 0.482 and 0.360 were located in the leaf extracts of *A. indicum, D. metel, L. inermis* and *A. indica* respectively on the TLC plate. Spot appeared bluish when sprayed with Folin Ciocalteau solution, which indicated the presence of phenolic compounds. Maximum antifungal activity against *A. alternata* was detected in the compound eluted from *A. indicum* and *D. metel* by preparative TLC which showed the inhibition zone of 4.90 and 4.80 mm, respectively whereas the compounds isolated from *L. inermis* and *A. indica* did not show any antifungal activity (Plate 8).

In order to separate alkaloids from leaf extracts, chloroform: methanol (15:5) as solvent and Dragendorff reagent was used. The presence of one spot was noticed in *A. indicum* and *D. metel* with Rf values of 0.880 and 0.972 under spray reagent.

Marked antifungal activity was shown by the compounds eluted from region corresponding to the above spot in *A. indicum* and *D. metel.* The antifungal activity was exhibited by inhibition of the mycelial growth of 6.80 and 5.20 mm, respectively in *A. indicum* and *D. metel* (Table 9; Plate 9).

4.9. Induction of defense-related enzymes by biocontrol agents and plant products

4.9.1. Changes in the Phenylalanine ammonia lyase (PAL) activity in groundnut plants

Generally the activity of phenylalanine ammonia lyase increased significantly up to five days in all the treatments and thereafter declined. The plants treated with biocontrol agents/ plant products following challenge inoculation with *A. alternata* significantly increased PAL activity. Maximum PAL activity was observed in Pf1 treated plants followed by challenge inoculation (192 nmol of transcinnamic acid mg^{-1}). This was followed by treatment with biocontrol agents / plant products alone. The plants inoculated with the pathogen also increased the PAL activity significantly up to 5 days (Table 10; Fig. 1).

4.9.2. Changes in the Peroxidase (PO) activity in groundnut plants

The peroxidase activity increased significantly up to five days in all the treatments and thereafter it declined. Treatment with biocontrol agents/ plant products challenged inoculation with the pathogen, maximum peroxidase activity was observed and the maximum being in Pf1 treated plants by recording 374 changes in absorbance min^{-1} mg^{-1} (Table 11; Fig. 2).

4.9.3. Changes in the Polyphenol oxidase (PPO) activity in groundnut plants

The polyphenol oxidase activity was increased significantly up to five days after pathogen inoculation in all the treatments and thereafter declined. The PPO activity was maximum in plants treated with biocontrol agents and plant products. Further, an increased activity of PPO was observed in the plants treated with biocontrol agents / plant products followed by challenge inoculation with *A. alternata.*

The higher polyphenol oxidase activity of 23.40 (changes in absorbance/min/mg) was recorded in plants treated with Pf1 and challenged inoculation with the pathogen, *A. alternata* (Table 12; Fig. 3).

4.9.4. Changes in the Chitinase activity in groundnut plants

The activity of chitinase increased significantly up to five days in all the treatments and thereafter it declined. In general, the plants treated with biocontrol agents/ plant products challenge inoculated with *A. alternata* recorded significantly the higher chitinase activity, followed by treatment with biocontrol agents/ plant products alone, compared to healthy plants. The maximum chitinase activity observed in plants treated with Pf1 challenge inoculation with the pathogen, *A. alternata* (16.20 n mol of GluNac/min/g) (Table 13; Fig. 4).

4.9.5. Changes in the β-1, 3-Glucanase activity in groundnut plants

The activity of β-1, 3 glucanase increased significantly up to five days in all the treatments and thereafter declined. The plants treated with biocontrol agents/ plant products recorded significantly higher β-1, 3-glucanase activity. In plants treated with biocontrol agents/ plant products, followed by challenge inoculation with the pathogen, the activity was further increased significantly.

The β 1-3 glucanase activity was significantly higher after 5 days (68 µg of glucose released /mg) in plants treated with Pf1 after challenge inoculated with the pathogen *A. alternata*, followed by Pf2, Bs1 and Bs5. When compared to plant products, the activity was more in biocontrol treated plants (Table 14; Fig. 5).

4.9.6. Changes in the Tyrosine Ammonia Lyase (TAL) activity in groundnut plants

The results showed that the activity of tyrosine ammonia lyase (TAL) increased significantly up to five days in all the treatments and thereafter declined. The plants treated

with biocontrol agents/plant products, followed by challenge inoculation with *A. alternata* recorded significantly higher TAL activity, followed by treatment with biocontrol agents/leaf extracts alone. The maximum TAL activity (23.13 nmol changes in absorbance/ min / mg of leaf tissue) was observed in plants treated with Pf1, followed by challenge inoculation with the pathogen *A. alternata* (Table 15; Fig. 6).

4.9.7. Changes in the phenolic content in groundnut plants

The results revealed that the phenolic content increased progressively up to five days in all the treatments and thereafter declined. The plants treated with biocontrol agents/ plant products followed by challenge inoculation with *A. alternata* recorded significantly higher accumulation of phenolic content. This was followed by treatment with biocontrol agents and inoculation with pathogen (*A. alternata*).

However, the maximum phenol content of 224.43µg of protein mg^{-1} of fresh weight of tissue was recorded in plants treated with Pf1, followed by challenge inoculation with the pathogen *A. alternata* (Table 16; Fig. 7).

4.9.8. Changes in total soluble protein content in groundnut plants

The results showed that the total protein content increased progressively up to five days in all the treatments and thereafter declined. The plants treated with biocontrol agents/ plant products, followed by challenge inoculation with *A. alternata*, recorded significantly higher protein content. Maximum protein content of 0.93 µg of protein mg^{-1} of fresh weight was recorded in plants treated with Pf1 followed by challenge inoculation with the pathogen *A. alternata* (Table 17; Fig. 8).

4.10. Native gel electrophoresis

4.10.1. Peroxidase

Native PAGE analysis revealed that PO2 isoform was induced in all treated plants. The expression of isoforms was more prominent in Pf1 and Bs1 pretreated plants and challenged with *A. alternata.* An additional isoform of PO1 was seen in biocontrol treated plants and challenged with the pathogen. Plants treated only with biocontrol agents did not show any additional PO isoform (Plate 10).

4.10.2. Polyphenol oxidase

Native PAGE analysis revealed that two PPO isoforms such as PPO1 and PPO2 were present in all the treatments. However, the plants treated with biocontrol agents and challenged with *A. alternata* showed prominent induction than the healthy and inoculated control. Three additional isoforms of PPO3, PPO4 and PPO5 were induced in the plants pretreated with biocontrol agents and challenged with *A. alternata* (Plate 11).

4.10.3. Protein

The results of the SDS-PAGE revealed that pre-treatment of groundnut plants with biocontrol agents induced ~14 and 25 kDa proteins. Plants treated with biocontrol agents followed by challenge inoculation with *A. alternata* induced additional proteins with molecular weight of 35, 40, 55 and ~ 97 kDa. Healthy and inoculated plants showed ~ 14 and 25 kDa protein induction (Plate 12).

4.11. Effect of biocontrol agents and plant products against *A. alternata* in pot culture condition

The effective biocontrol agents *viz.,* Pf1, Pf 2, Bs1 and Bs5 and leaf extracts *viz.*, *A. indicum* and *D. metel* were selected based on the effectiveness in reducing the growth of the pathogen under *in vitro* condition. The results revealed that all the treatments were effective in reducing the disease intensity compared to control.

However, Pf1 was most effective in reducing disease index of 24.17 per cent, followed by Pf 2, Bs1 and Bs5 (seed treatment plus foliar spray) with 26.21, 27.13 and 28.21 per cent, respectively. The foliar spray of biocontrol agents recorded lesser disease incidence than spraying with plant products. The growth parameters like plant height and number of leaflets per plant increased in biocontrol agents delivered through seed treatment plus foliar spray (Table 18 and 19).

4.12. Efficacy of biocontrol agents against leaf blight caused by *A. alternata* in field conditions

Three field trials were conducted at Coconut Research Station, Aliyar Nagar, during 2002-´03 and 2003-'04, to assess the effect of various biocontrol agents on leaf blight disease of groundnut. The results of Rabi seasons (2002-'03) revealed that all the biocontrol agents effectively reduced the disease intensity, compared to control. Pf1 as seed treatment and foliar spray on 30 and 45 DAS recorded the lowest disease index of 23.23 per cent with higher yield of 1342 kg ha^{-1} as against higher incidence of 59.23 per cent and lowest yield of 940 kg ha^{-1} in control (Table 20; Fig. 9).

The second trial conducted during Kharif 2003-'04 also revealed that the Pf1 was most effective by recording lesser disease incidence of 25.47 per cent with highest yield of 1342 kg ha^{-1} as against 61.93 per cent disease incidence and lowest yield of 1012 kg ha^{-1} in control (Table 21; Fig. 10).

In the third trial conducted during Rabi 2003-'04 also, Pf1 recorded lesser disease index of 23.24 per cent with highest yield of 1412 kg ha^{-1} as against highest disease index of 48.31 per cent in control with lowest yield of 1033 kg ha^{-1} (Table 22; Fig. 11; Plate 13).

4.13. Survival of biocontrol agents

4.13.1. Survival of *P. fluorescens* and *B. subtilis* on phyllosphere

Foliar application of talc based formulation of Pf1 and Bs1 led to increase in phyllosphere population. The maximum population (8.3 cfu x10^8 /g) was observed after 15 days spraying and thereafter the population decreased. In *B. subtilis* also, the maximum population (8.1 cfu x 10^8/g) was recorded at 15 days after sowing and thereafter decreased (Table 23).

4.13.2. Population dynamics of *P. fluorescens* and *B. subtilis* on groundnut phyllosphere, under green house condition

The maximum population of Pf1 and Bs1 was recorded on zero day of storage. As the period increases, the bacterial population was found to be decreased. However, the Pf1 and BS1 were found to survive in talc based formulation upto 90 days with the maximum population of 7.9 and 7.8 cfu x 10^8 g^{-1} respectively (Table 24).

4.14. Detection of fungal pathogen by DAC - ELISA

4.14.1. Determination of titre value of the antiserum against Alternaria leaf blight

The titre value of the antiserum raised against *A. alternata* was determined as described in Table 25, using different dilutions of antigen and antiserum. The titre value was 1.231, when the antibody dilution of 1:1000 and antigen dilution of 1:100 were used. Hence, the optimum dilution for antiserum and antigen was 1:1000 and 1:100 respectively (Table 25; Plate 14).

4.14.2. Detection of Alternaria leaf blight fungus using polyclonal antisera in groundnut leaf

The results of the experiments revealed that the presence of *A. alternata* was detected on 4^{th} day after inoculation indicated that the disease can be detected 6 days

before the symptom expression, where the natural symptom appears 10 days after artificial inoculation (Table 26; Plate 15).

4.15. Epidemiological studies

4.15.1. Influence of weather parameters on Alternaria leaf blight disease incidence

4.15.1.1. Rabi, 2002-'03

The results on the disease revealed that the *A. alternata* occurred at 34, 32 and 30 days after sowing in first, second and third sowing respectively. In first sowing, there was a positive correlation between maximum temperature, minimum temperature, GDD and Alternaria leaf blight disease incidence. While in the second sowing, minimum temperature, GDD and RH (e) were significantly associated with the disease incidence. Minimum temperature and evening RH showed positive association with the disease in the third sowing. While viewing the results comprehensively, minimum temperature showed positive correlation during all the three sowings. The first and second sowing showed positive correlation for GDD. Such performance was also noted for RH (e) during second and third sowing (Table 27 and 28).

The study on multiple regression revealed a significant relationship and the developed equations are given in Table 30. The equation component indicated that in respect of first sowing, minimum temperature followed by evening RH had contributed greatly for disease development (Y). In second sowing minimum temperature followed by growing degree days contribute greatly for disease development. In respect of third sowing, GDD and minimum temperature had contributed greatly to the disease development (Table 29 and 30).

4.15.1.2. Kharif, 2003-'04

The data on the disease incidence showed the disease occurred on 34, 35 and 33 days after sowing in first, second and third sowing respectively in kharif season. There was positive correlation for DV during first sowing and morning RH for second sowing. Interestingly all the weather parameters had no association for the disease incidence during third sowing. In the regression equations, the DV had significant contribution for the development of disease incidence during first sowing, while morning RH had contributed for second sowing (Table 31 to 34).

4.15.1.3. Rabi, 2003-'04

The results revealed that the disease occurred at 21, 39 and 38 days after sowing in first, second and third sowing respectively. The minimum temperature and GDD had positive correlation with the disease incidence in all the three sowings evaluated. In addition, maximum temperature, bright sunshine hours had positive correlation with the disease incidence for first sowing. The maximum temperature and evening RH have contributed for the disease incidence respectively for second and third sowing. The regression equation had attained significance statistically for first sowing and the greatest contribution was obtained from GDD followed by maximum temperature and minimum temperature. In this component though GDD contributed more, it was on the negative side. For second sowing, a similar trend was obtained and the contribution from GDD was positive while minimum temperature and maximum temperature contribute negatively. In respect of third sowing, there was good contribution on positive side for evening RH and minimum temperature while negative contribution for GDD (Table 35 to 38).

CHAPTER V

DISCUSSION

Groundnut (*Arachis hypogaea* L.) leaf blight incited by *Alternaria alternata* (Fr.) Keissler was reported from several countries. In India, the research work on this disease is very limited, though it occurs in severe proportion. Hence, the present study was undertaken on survey, identification of the pathogen, epidemiological factors responsible for disease outbreak, detection of the disease by ELISA, management of the disease using non-chemical means and induced systemic resistance. The results obtained are discussed herein.

5.1. Survey, identification of the pathogen and its pathogenicity

A detailed survey was made to observe the incidence of leaf blight disease of groundnut caused by *A. alternata*, during Rabi 2003. The results showed that the disease occurred in severe form almost in all groundnut growing districts of Tamil Nadu, where the per cent disease index ranged from 33.80 to 62.00. The pathogen isolated from the diseased tissues, by artificial inoculation produced typical symptoms as that of naturally infected plants *viz.*, appearance of yellowing symptoms at the apical portion of the leaves, spreading towards the petiole, followed by the lesions turning into light to dark brown colour and blighting of leaves. Vein blighting symptom was also observed in the present study. Similar type of symptoms were reported by several workers (Aulakh, 1969; Kulkarni, 1974; Ghewande *et al.*, 1982). However, Bohovik (1936) did not observe any necrosis of veins and veinlets. In the present study, the pathogen associated with leaf blight symptom was identified as *Alternaria alternata* (Fr.) Keissler at IARI, New Delhi.

5.1.1. Isolation of antagonistic microflora

Various strains of *P. fluorescens* and *B. subtilis* were frequently obtained from the rhizosphere of groundnut plants grown in different parts of Tamil Nadu. However, biocontrol efficacy of the strains varied due to inherent potential of the strains that suppressed the pathogen (Sakthivel *et al.*, 1986; Rosales *et al.*, 1993). Fluorescent pseudomonads are known to aggressively colonize the roots therefore they are efficient competitors and persist throughout the crop season (Kloepper and Schorts, 1978). The present study indicated that majority of the strains isolated from rhizosphere of the groundnut were *P. fluorescens* and *B. subtilis*.

5.2. Effect of bacterial antagonists on radial mycelial growth of *A. alternata*

Selection of suitable, effective strain of biocontrol agents is of prime importance in biological control. In the present study under *in vitro* condition, strains of *P. fluorescens* (Pf1 and Pf2) and *B. subtilis* (Bs1 and Bs5) recorded maximum reduction in mycelial growth of *A. alternata* with higher inhibition zone. The effectiveness of *P. fluorescens* and *B. subtilis* in inhibiting radial mycelial growth of *Alternaria* spp. have been reported by several workers. The radial mycelial growth of *A. porri*, a pathogen for onion blight was drastically inhibited by Pf1 (Ravi 2000). Renuka (2003) reported that Pf1 and *B. subtilis*, significantly inhibited the mycelial growth of *A. chlamydospora*. Similar observation was also recorded on *A. helianthi* by Sivagamy (2003).

The inhibitory effect of *Pseudomonas* and *Bacillus* strains against *A. alternata* might be due to the production of antibiotic substances. Several strains of *Pseudomonas* spp. and *Bacillus* spp. have been reported to produce wide array of antibiotics *viz*., 2,4 diacetylphloroglucinol, oligomycin, oomycin A, phenazine, pyoluteorin, pyrolnitrin, pyocyanin, iturin, surfactin and several other uncharacterized

molecules (Kim *et al.,* 1989; Keel and Defago, 1997; Whipps, 1997 and Nielson *et al.,* 1998).

5.3. Effect of leaf extracts on radial mycelial growth of *A. alternata*

The results of the present study clearly indicated that the leaf extracts of *Abutilon indicum* and *Datura metel* (10%) were highly effective in reducing the radial mycelial growth of the pathogen. Similar effect of various plant products against *Alternaria* spp. have been reported by several workers. Renuka (2003) reported that the extract of *A. indicum* and *D. metel* showed maximum reduction in mycelial growth of *A. chlamydospora*. The bulb extract of *Allium sativum* drastically reduced the mycelial growth of *A. helianthi* in sunflower (Sivagami, 2003). The extracts of *Mimosa* bark and gallnut powder inhibited the development of *A. alternata* with an inhibition zone of 21 and 15 mm, respectively (Metin *et al.,* 1999). The inhibition of mycelial growth might be due to the presence of antifungal components in the extracts (Ansari, 1995; Meena *et al.*, 1999; Sateesh, 2001; Anusha, 2003).

5.4. Antifungal metabolites produced by *Pseudomonas* sp.

Indole acetic acid (IAA) salicylic acid, HCN and siderophore production etc., are some of the mechanisms involved for the antagonistic activity of fluorescent pseudomonads against soil borne pathogens (Wei *et al*., 1991). The present study indicated that the production of IAA, salicylic acid and siderophores was maximum in Pf1. In case of HCN, Pf1 and Pf2 recorded maximum activity followed by other strains *viz*., Pf3, Pf4 and Pf5. The role of secondary metabolites of fluorescent pseudomonads in the induction of resistance was reported by several workers (van Loon *et al.,* 1998; Haas *et al.,* 2000). Among the different secondary metabolites of fluorescent pseudomonads, siderophores and salicyclic acid are implicated in the induction of systemic resistance (Maurhofer *et al* 1994). De Meyer and Hofte (1997) reported the role of salicylic acid produced by *P. aeruginosa* 7NSK 2 as a

determinant of inducing systemic resistance. The involvement of HCN in the suppression of pathogen was reported by several workers in different hosts (Stutz *et al.*, 1986; Defago *et al.*, 1980; Weststeijin, 1990; Wei *et al*., 1991; Meena, 2000) from soil pseudomonads. Water-soluble siderophores were involved in disease suppression. Siderophore mediated disease suppression by *P. fluorescens* has been well documented (Loper and Buyer, 1991; Meena, 2000).

5.5. Antifungal substance in plant extracts

Phenols and alkaloids are known for their direct toxicity to fungal pathogens (Cascaraes *et al.*, 1986). The present study revealed that phenolic and alkaloid compounds separated from *A. indicum, D. metel* were highly inhibitory to the growth of *A. alternata.*

Grag and Siddiqui (1992) reported that the phenol 1,8-cineole isolated from *Luvunga scandens* was highly inhibitory to the growth of *A. alternata*. Naidu (1988) studied the presence of chlorogenic acid from *Codiaeum variegatum* and found to be active against *A. alternata.* Hiraoka *et al.* (1996) separated tropane alkaloid like scopolamine and hyoscyamine from *D. metel*. Two alkaloids were isolated from the seeds and identified on the basis of spectral data, which were found to be lysicamine and juzirine (Yin *et al.*, 1997). Doepke *et al*. (1975) isolated antiviral glycoalkaloids from the leaves of *Solanum torvum*.

5.6. Induced systemic resistance

Besides direct antagonistic activity by the production of various bacterial metabolites, induction of systemic resistance by fluorescent pseudomonads against diseases has been established as a new mechanism by which the plants defend themselves from pathogen attack (van Peer *et al.*, 1991; van Loon *et al.*, 1998). Induced resistance in several crops is associated with enhancement of lignification and also increased activities of enzymes involved in phenyl propanoid pathway and PR protein synthesis (Boller and Mauch,

1988; Hammerschmidt and Kuc, 1995). Recent studies implies that prior application of fluorescent pseudomonads strengthen the cell wall structures resulting in restriction of pathogen invasion in plant tissue (Benhamou *et al.,* 2000; Chen *et al.,* 2000).

5.6.1. Phenylalanine Ammonia Lyase (PAL)

Phenylalanine ammonia lyase plays an important role in the biosynthesis of various defense chemicals in phenyl propanoid metabolism (Daayf *et al.,* 1997). The results of present study revealed that all biocontrol agents and plant products induced the plants to synthesize more amount of PAL. The maximum PAL activity was observed in 5^{th} day in plants treated with *P. fluorescens* (Pf1) and challenge inoculated with the pathogen. The plants treated with the pathogen alone also showed increased PAL activity. Similar reports have been reported by several workers.

P. fluorescens induced PAL activity in rice (Meena *et al.,* 1999; Radja Commare, 2000) and groundnut (Meena, 2000). Induction of PAL by fluorescent pseudomonads was reported in cucumber against *Pythium aphanidermatum* (Chen *et al.,* 2000) and bean against *Botrytis cinerea* (Zdor and Anderson, 1992). The treatment with plant extracts led to an increase in activities of PAL in rice plants by sheath blight pathogen as reported by Sateesh (2001). Similarly, Renuka (2003) reported PAL activity increased by the application of biocontrol agents and plant products followed by challenge inoculation with *A. chlamydospora* infecting chrysanthemum.

5.6.2. Peroxidase and polyphenol oxidase

Peroxidase represents another component of an early response in response to pathogen attack and play a key role in the biosynthesis of lignin, which limit the extent of pathogen spread (Bruce and West, 1989). The products of this enzyme in the presence of hydrogen donor and hydrogen peroxide have antimicrobial activity and even antiviral activity (van Loon and Callow, 1983). In the present study, earlier and increased

peroxidase activity has been recorded in *P. fluorescens* Pf1 treated plants challenged with the pathogen. In bean, rhizosphere colonization of various bacteria induced the peroxidase activity (Zdor and Anderson, 1992). Chen *et al*. (2000) reported higher PO activity in cucumber roots treated with *P. corrugata* challenged with *P. aphanidermatum*.

Native PAGE analysis revealed that addition of PO1 isoform was induced in groundnut plants pretreated with biocontrol agents and challenge inoculated with *A. alternata*. Plants pretreated with Pf1 and Bs1 and challenged with the pathogen showed higher intensity of PO1 isoform than the others. The plants treated with biocontrol agents alone did not show the PO1 isoform.

Similar to other enzymes, PPO activity was increased in groundnut plants treated with biocontrol agents and plant products at 5 days after treatment. The PPO activity was still higher in treated plants challenge inoculated with the pathogen. Five isoforms of PPO (PPO1-PPO5) were observed in the plants treated with biocontrol agents and challenged with *A. alternata* while plants treated with biocontrol agents alone recorded only 2 isoforms of PPO1 and PPO2. The higher level of expression of PPO1 and PPO2 might have induced defense responses. Polyphenol oxidase oxidizes phenolics to highly toxic quinines and are speculated to be involved in the terminal oxidation in the diseased plant tissue which was attributed for its role in disease resistance (Kosuge, 1969). Earlier, Meena (2000) reported the induction of PPO isoforms in plants pretreated with *P. fluorescens* and challenged with late leaf spot pathogen *C. personatum*. Similarly Renuka (2003) reported that the PO and PPO activity were increased by the application of biocontrol agents and plant products challenged with *A. chlamydospora* infecting chrysanthemum. Chen *et al.* (2000) reported that various rhizobacteria and *P. aphanidermatum* induced the PPO activity in cucumber root tissues.

5.6.3. Chitinase and β-1,3 glucanase

In general, fungal cell contain chitin and glucan as their cell wall constituents. The main modes of action of antagonistic microbes are production of lytic enzymes (chitinase and β-1, 3 glucanase), which act on cell wall of organisms, which have chitin and glucan as their cell wall component (Sing *et al.*, 1999) and through induced systemic resistance (ISR) in plant system. In the present study, the activity of chitinase and β-1, 3 glucanase was higher in biocontrol agents treated plants challenged with *A. alternata* indicating that it might have resulted in the lysis of invading pathogen.

The activity in Pf1 treated plants was about two fold at five days after inoculation compared to control plants. Due to challenge inoculation with *A. alternata*, β-1, 3-glucanase activity increased about three fold in Pf1 treated plants. This result indicated the possibility of involvement of β-1-3-glucanase in the defense mechanisms against leaf blight of groundnut. A direct role for β-1, 3-glucanases in defense against pathogens have been proposed because the substrate of these enzymes is a major component of the cell wall of many fungi (Lim *et al.*, 1991; Fridlender *et al.*, 1993).

Earlier Viswanathan and Samiyappan (1999) reported that ISR by fluorescent pseudomonads, which were associated with induction of chitinase, as a promising technology for the management of red rot of sugarcane. Plants respond to pathogen infection by producing a number of proteins believed to be important in protecting them from the deleterious effects of the pathogen (Lamb *et al.*, 1989). These include enzymes capable of hydrolyzing structural components of the pathogen. Chitinases are enzymes that hydrolyse the N-acetyl glucosamine polymer chitin and these are present in tissues of a broad range of crop and non-crop species (Colligne *et al.*, 1993). Increase in chitinase activity has been reported in bean against *Colletotrichum lindemuthianum* and in barley against *Erysiphe graminis* (Toyoda *et al.*, 1991).

5.6.4. Tyrosine Ammonia Lyase (TAL)

Induction of tyrosine ammonia-lyase (TAL) by the application of Pf1 and Bs1 formulations and plant products were observed during the present study. TAL activity paved the way for a secondary entry into the phenyl propanoid pathway effectively bypassing the 4-hydroxyl cinnamic acid to p-coumaric acid. The flux of material *via* the TAL route was thought to be secondary and most metabolites entered the general phenyl propanoid pathway through PAL (Barber and Mitchell, 1997; Meena, 2000). Antimicrobial properties of plant derivatives have long been recognized as an important factor in disease resistance (Mahadevan, 1982). Moreover these natural plant products are largely non-phytotoxic, environmentally non-pollutive, easily decomposable due to their organic origin, renewable and also abundantly available (Gulter, 1998). In the present investigation, TAL activity was increased in all the treatments significantly when compared to control.

5.6.5. Total phenol

The phenolic compounds may contribute to enhance the mechanical strength of host cell wall and may also inhibit the fungal growth, as phenolics are fungitoxic in nature. Seed treatment with *P. fluorescens* 63-28 induced the accumulation of phenolics in tomato root tissues (M' Piga *et al*., 1997). The hyphae of the pathogen surrounded by phenolic substances exhibited considerable morphological changes including cytoplasmic disorganization and loss of protoplasmic content. Accumulation of phenolics by prior application of *P. fluorescens* in pea has been reported against *P. ultimum* and *F. oxysporum* f.sp. *pisi* (Benhamou *et al*., 2000). The present study also indicated that the higher level of accumulation of phenolics was observed in biocontrol agents treated plants and challenge inoculated with the pathogen. Similar findings were reported in rice against *R. solani* (Meena *et al*., 1999; Radja Commare, 2000) and sugarcane against *Colletotrichum falcatum* (Viswanathan and Samiyappan, 1999). Similarly Meena (2000) reported that a three fold increase in phenolic content was seen in groundnut leaves

pretreated with *P. fluorescens* and challenge inoculated with *C. personatum*. Renuka (2003) reported that phenolic compounds increased due to the application of biocontrol agents and plant products challenged with *A. chlamydospora* affecting chrysanthemum.

Thus induction of defense enzymes involved in phenylpropanoid pathway by biocontrol agents against pathogen infection leads to induced protection by synthesizing various defense compounds. Earlier and enhanced levels of peroxidase, polyphenol oxidase, chitinase, β-1, 3 glucanase, TAL, phenolics and proteins by application of biocontrol agents suppressed the further infection of *A. alternata* in groundnut plants.

5.7. Disease management using biocontrol agents

Plant growth promoting rhizobacteria play a vital role in the management of various fungal diseases. But one of the major hurdles experienced with biocontrol agents is the lack of appropriate delivery system. In the present study, application of Pf1 and Bs1 through seed treatment and foliar spray gave maximum control of leaf blight, under pot culture and field conditions. The efficacy of biocontrol agents to reduce leaf blight disease might be due to the production of salicylic acid, antibiotics, lytic enzymes and the induction of defense related enzymes like PAL, PO, PPO, chitinase, glucanase, TAL, phenols and proteins.

Fluorescent pseudomonads and *B. subtilis* belong to a major group of rhizosphere dwelling bacteria known as plant growth promoting rhizobacteria (PGPR) (Kloepper *et al.*, 1980; De Fereitas and Germida, 1991; Hofte *et al.*, 1991; Leeman *et al.*, 1995a; Liu *et al.*, 1995a,b, c). However, few studies have been carried out on the management of foliar disease with fluorescent pseudomonads (Mew and Rosales, 1986; Knudsen and Spurr, 1987; Meena, 2000; Renuka, 2003). Several strains of *P. fluorescens* and *B. subtilis* strains were isolated from rhizosphere of groundnut (Ganesan and Gnanamanickam, 1987), soybean (Kloepper *et al.,* 1985), sunflower (Hebber *et al.,* 1991) and groundnut

(Meena, 2000). However, the antagonistic potential of different strains vary (Rosales *et al.*, 1986; Hagedorne *et al.*, 1993).

In the present study, *P. fluorescens* and *B. subtilis* strains were tested for their plant growth promoting ability. Among the tested strains, Pf1 recorded the higher germination percentage of 76.00 and increased shoot and root length of 17.90 and 11.30 cm respectively.

Seed treatment with fluorescent pseudomonads resulted in early seedling emergence and growth of plants in various investigations (De Freitas and Germida, 1991; Muthamilan, 1994; Meena, 2000). Vivekananthan *et al*., (2004) reported that preharvest application of *P. fluorescens* (FP7) with chitin formulation significantly reduced the anthracnose incidence upto 60 per cent over untreated control. Bharathi *et al.* (2003) reported that in case of chilli fruit rot and die back incited by *C. capsici*, foliar spray of *P. fluorescens* and *B. subtilis* were effective in reducing the disease. The beneficial effects of these bacteria, in most cases, have been related to their ability to produce plant growth hormones and antimicrobial substances and to protect growing roots from deleterious root microbes present in the rhizosphere (Lifshitz *et al.*, 1987; Schippers *et al.*, 1987; Weller, 1988; Meena, 2000).

The increase in biomass production by Pf1 and Bs1 (seed treatment plus foliar spray) may be due to the production of plant growth promoters or through indirect stimulation of nutrient uptake by producing siderophore or antibiotics to protect plant from deleterious rhizosphere organisms. van Peer and Schippers (1988) documented that increase in the root length and shoot length of tomato, cucumber, lettuce and potato as a result of bacterization with pseudomonads strains.

The strains that showed maximum inhibition of *A. alternata* under *in vitro* were tested for their efficacy in controlling leaf blight under green house condition. The Pf1 strain reduced the disease by 57.79 per cent over control when used as seed treatment and foliar spray. Similarly in groundnut plants, Pf1 as seed treatment and foliar spray gave maximum reduction of late leaf spot disease (Meena, 2000). The pod yield per plant was also found to be increased due to these treatments.

Earlier workers have used bacterial cell suspension for seed treatment, soil application or foliar spray for the control of foliar diseases. Liu *et al.* (1995a) reported that injection or other methods of application of bacterial suspension is impracticable for large-scale application to control foliar diseases in field (Capper and Higgins, 1993). The powder formulation with long shelf life would be beneficial. Several formulations of *P. fluorescens* had been developed for large-scale field application (Vidhyasekaran and Muthamilan, 1995; Vidhyasekaran *et al.*, 1997a,b). A talc-based formulation developed by Vidhyasekaran and Muthamilan (1995), was effective for the control of rice blast (Vidhyasekaran *et al.*, 1997a), pigeon pea wilt (Vidhyasekaran *et al.*, 1997b) and groundnut late leaf spot (Meena, 2000).

P. fluorescens and *B. subtilis* populations were found to be increased in phyllosphere on 5^{th} day after foliar application and reached maximum on 15 days after spraying and thereafter declined. The result clearly indicated that *P. fluorescens* and *B. subtilis* were able to multiply, utilizing the nutrients available on and in the leaves.

The present studies indicated the usefulness of talc based powder formulation of *P. fluorescens* and *B. subtilis* strains for the control of leaf blight of groundnut. This bacterium was observed to survive up to 90 days in the formulation with the required population (10^8 cfu/g). This formulation has practicable advantage as it can directly be supplied to the farmers. Seed at the rate of 10 g kg^{-1} of seed, and foliar spray at 0.2 per cent

concentration showed the lowest leaf blight intensity and increased the pod yield. Seed bacterization with biocontrol agents had been reported to control several diseases (Mew and Rosales, 1986; Lee *et al*., 1990; Wei *et al*, 1991; Tosi and Zazzerini, 1994; Meena, 2000).

When the treated seeds were sown, the bacterium established well in the rhizosphere of groundnut soil. The successful antagonist should colonize the rhizosphere at the time of seed germination itself. In other words, the antagonist should move from spermosphere to rhizosphere and establish there (Weller, 1983). Numerous reports on the control of foliar diseases with *P. fluorescens* and *B. subtilis* applied to foliage were reported. Fluorescent pseudomonads, which constitute 20 per cent of the total bacterial population, expected to give better protection against the pathogens. They have high affinity for amino acid exudates and probably this might have contributed to their high rhizosphere competence (Mew and Rosales, 1986; Gnanamanickam and Mew, 1992; Meena, 2000).

Efficacy of Pf1 formulation in the management of leaf blight has been demonstrated in three field trials. Seed treatment plus foliar spray was effective method of application of Pf1 formulation in controlling the disease. It might be greatly due to increased population of *P. fluorescens* and *B. subtilis*, which induced resistance. A threshold population density of *P. fluorescens* and *B. subtilis* strain had been shown to be required for significant suppression of late leaf spot of groundnut (Meena, 2000). A positive relationship between population size of *P. fluorescens* and *B. subtilis* and level of suppression of various diseases had been reported (Rabindran and Vidhyasekaran, 1996; Vidhyasekaran and Muthamilan, 1992). Seed treatment combined with foliar spray of Pf1 formulation increased the yield. The increase in groundnut pod due to application of Pf1 formulation might be associated with the decrease in disease incidence and increase in

plant growth due to plant growth promoting characteristics of fluorescent pseudomonads (Lifshitz *et al.*, 1987).

From the above studies, it could be concluded that Pf1 formulation is able to induce systemic resistance against foliar diseases. Several workers had also reported the induction of systemic resistance by *P. fluorescens* in different crops against various pathogens (Hoffland *et al.*, 1996; Leeman *et al*., 1995a,b; M'Piga *et al.*, 1997; Meena, 2000).

The present study revealed the potentiality of *P. fluorescens* (Pf1) talc based formulation for controlling leaf blight disease of groundnut. Studies conducted so far have thus reinforced the prospects of using this biocontrol agent on a commercial scale as a successful alternative for chemical control of foliar disease and have further implicated the role of *P. fluorescens* in the defense mechanism against the pathogen.

5.8. Detection of *A. alternata* by using ELISA

Fungal pathogens in general, are relatively easily diagnosed by the symptoms induced in infected plants and characteristics of spores and mycelium. In certain cases alternative methods like ELISA are required for their detection and identification. Currently, simple diagnostic kits are being developed to conduct plant "Site testing".

The current investigation revealed that *A. alternata* could be detected by ELISA using antigen dilution of 1:100 and antiserum dilution of 1:1000 with the titre value of 1.181. The pathogen was detected from groundnut plants 4 days after inoculation i.e. 6 days before the symptom expression using ELISA. Similar results were reported by several workers. *P. fragariae* var. *rabi* was detected in root tissues of raspberry using a commercial multiwell assay kit from 4 days after inoculation (Olsson and Heiberg, 1997). *Phytopthora infestans*, causing potato late blight disease was detected by indirect ELISA

before the appearance of first symptoms. The pathogen could be detected in potato shoots of 5-9 weeks old plants about 39 days before disease outbreak in the field (Schlenzig *et al.,* 1999). *C. gloeosporioides* infection in banana fruits were detected by ELISA even before the symptom expression (Sible, 2003). Kandan (2003) also reported the detection of *Ganoderma lucidum* infection in coconut trees 75 days before symptom expression by using polyclonal antiserum raised against the pathogen. *Phomopsis* spp. was detected in decayed soybean using polyclonal antibodies developed against *Phomopsis* spp. (Velichetti *et al.,* 1993). Viswanathan *et al.* (1998) found that the PCAbs raised against a 101 kDa polypeptide and unfractionated proteins of *Colletotrichum falcatum* gave positive reaction.

The use of an immuno assay and PCR based detection will help in the detection of seed borne infection of *A. alternata* in the seed of groundnut and helps to adopt suitable management strategies against seed borne *A. alternata* disease in groundnut seeds.

5.9. Epidemiological studies

The trial on epidemiology was conducted in three seasons within two years of study *viz*., Rabi 2002-'03, kharif 2003-'04 and Rabi 2003-'04.

In respect of the weather that prevail during a season may get varied and hence variability in weather parameters is a rule rather than an exception. Weather variability had been reported already by many meteorologists both in India and abroad. During Rabi 2002-'03 maximum temperature ranged from 26.5°C to 37°C while it was from 12.5°C to 28.4°C for minimum temperature. Morning RH was more than 85 per cent while evening RH went upto 73 per cent. From the correlation study made for all the three sowing dates, minimum temperature had positive correlation with disease incidence and thus it is inferred that for every degree increase in minimum temperature there must be an increase in the disease

incidence. The minimum temperature of a particular area is controlled by re-radiation of the solar energy received by the earth which occurs normally at night. The disease incidence during Rabi 2002-'03 was upto a maximum level of 54.21 PDI. Based on the result with increase in minimum temperature there might be warm environment that occurred during night which might have triggered the disease incidence and hence irrespective of the sowings studied there was positive correlation between minimum temperature and disease incidence. In the present investigation also this happened.

Similarly for I and II sowings, GDD did influence the disease incidence while RH(e) did influence the disease in second and third sowing respectively. The GDD normally decides the phenological stages of crop species. In the present results also was to observed that the life cycle of pathogen also dictated by GDD. Whenever the evening RH went beyond 60 per cent and continued to be 60 per cent for more than three nights there was evident indicating that the leaf wetness would increase simultaneously. This situation was favourable for the falling conidia from the transporter wind and hence the spores might have germinated and well established. This relationship was found in the present investigation. Confirmative influence of GDD and minimum temperature was also observed for this disease incidence during Rabi 2003-'04 in the present investigation.

In respect of kharif 2003-'04 there was an exception that Diurnal Variation (DV) had positive correlation in first sowing and morning RH in the second sowing. This event was not an exception. Diurnal variation is nothing but the difference between maximum temperature and minimum temperature and it may occur in two extremes of temperature viz., on the upper range and also on the lower range.

During kharif 2003-'04 the diurnal variation ranged from 6 to 15.80 during the crop growth period. Except few days of crop growth the diurnal variation was more than

10 in all the growing period especially after 30 days from sowing. These might have favoured the disease incidence. This must be the first finding reported from this thesis since diurnal variation is a derived weather parameter, it might have escaped from the eyes of the past scientists.

Information on the epidemiology of leaf blight of groundnut caused by *A. alternata* is sparse. Previous research on this area also revealed that Alternaria spore concentrations were positively correlated with maximum temperature and negatively correlated with precipitation (Hervero and Zaldivar, 1997). Linear regression analysis indicated that the amount of rainfall, duration of leaf wetness and average daily temperatures were positively correlated with disease while it was negatively correlated to the number of conidia trapped. Zheng and Sutton (1994) reported that 84 h leaf wetness at 15-32°C favoured the development of lesions caused by *Diplocarpon earlianum* in strawberry leaves. Uddin *et al*. (2002) reported that grey leaf spot development in rye grass turf is positively correlated with different temperature of 20, 24, 28 and 32°C and different leaf wetness duration.

Srikanta Das and Das (2000) reported that in stepwise multiple regression equation in the rust disease of groundnut, maximum temperature, minimum temperature, maximum relative humidity and minimum relative humidity had positive correlation in all the three (rainy, winter and summer) seasons. In case of groundnut rust, disease severity was inversely correlated with maximum temperature, relative humidity, rainfall and wind velocity (Srikantha Das *et al*., 1996). Salako and Olorunju (1987) reported that in case of rust disease of groundnut was correlated with amount and spread of rainfall. Paria and Raj (2000) reported that the groundnut rust disease was positively influenced by temperature and RH and rainfall had no significant role.

Lokhande *et al*. (1998) reported that temperature had negative correlation while RH was positively correlated with rust disease of groundnut. Late leaf spot of groundnut was positively correlated with minimum temperature, maximum RH and rainfall while the disease severity was more when the maximum temperature decreased (Adiver *et al*., 1998) and the rust and leaf spot diseases of groundnut had positive correlation with rainfall, RH and temperature (Hazarika *et al*., 2000).

Moss and Trevathan (1987) studied the different environmental factors conducive for infection of rye grass by *P. grisea.* They reported that 26°C was most favourable for grey leaf spot development. Patil *et al*. (1988) studied the effect of different temperatures on the growth of *A. tenuissima* and among various temperatures tested, 25°C was most favorable for the growth of the pathogen. Evans *et al*. (1992) stated that the Alternaria leaf blight disease severity was increased with increasing wetness duration of 2 to 24 h at 12-30°C. The optimum temperature of 18°C and leaf wetness duration of 8h were required for disease development.

Corson (1985) indicated positive correlation of Alternaria blight with yield in sunflower. Latin (1992) developed the empirical models to describe an observed relationship between Alternaria leaf blight and yield loss in muskmelon. Latin *et al*. (1994) studied the relationship between epidemic severity of Alternaria leaf blight and quantitative representations of the change in soluble solids content (SSC) during sampling period. Results showed that SSC decreased with increased severity of Alternaria leaf blight epidemics.

The present study indicated that, the leaf blight caused by *A. alternata* occurs widely in Tamil Nadu wherever groundnut is grown and the needs for the management of the disease. Though fungicides are effective for the management of the disease, due to

uneasiness in their application, high cost, environmental risk, human health hazards and development of fungicide resistant strains, their use is being discouraged. But the biocontrol agent *viz*., *P. fluorescens* Pf1 is easy to apply as seed treatment and foliar spray without any undesirable characters as in chemical fungicides. Once it is applied, it survives and persists for a longer period and protects the plants from pathogen attack (van Loon *et al*., 1998). Broad-spectrum of ISR and induction of multigenic defense system encoding diverse proteins by *P. fluorescens* is another advantage in which there will not be any development of resistant strains. From the present study, it can concluded that, management of groundnut Alternaria leaf blight by using Pf1 strain of *P. fluorescens* is highly effective, economical and eco-friendly and it can be included into the integrated disease management strategy.

CHAPTER VI
SUMMARY

- The survey conducted in ten major groundnut growing districts of Tamil Nadu during Rabi 2003 revealed that the maximum Alternaria blight incidence was noticed in all the districts surveyed viz., Cuddalore, Salem, Coimbatore, Madurai, Pudukottai, Villupuram, Erode, Dharmapuri, Thiruvannamalai and Trichirapalli ranging from 33.77 to 63.22 per cent disease index.
- Among the five *P. fluorescens* strains and ten *B. subtilis* strains tested under *in vitro* conditions for the inhibition of mycelial growth of *A. alternaria* Pf1 and Bs1 strains significantly recorded the maximum inhibition in mycelial growth.
- Among the sixteen plant species tested, *A. indicum* significantly recorded the maximum inhibition the mycelial growth of *A. alternata* followed by *D. metel.*
- Seed treatment with Pf1 significantly showed higher germination percentage root and shoot length resulting in increased vigour index in groundnut compared to Pf2, BS 1 and BS5 strains.
- Studies on the production of inhibitory metabolites by fluorescent pseudomonads showed that among five strains of *P. fluorescens* Pf1 strain produced maximum amount of IAA, salicylic acid, siderophores and HCN content.
- The leaf extracts of *A. indicum* and *D. metel* by preparative TLC revealed phenolic and alkaloid compounds which were responsible for the antifungal activity against *A. alternata* and they also had maximum content of phenol and soluble protein content.
- The groundnut plants treated with biocontrol agent / plant products and challenge inoculated with *A. alternata* recorded significantly increased activity of PAL, PO, PPO, TAL, chitinase and β1,3 glucanase activity and higher level of accumulation

of phenol and protein content. Among them Pf1 strain has recorded maximum activity of these above enzymes.

- Expression of PO2, PPO1 and PPO2 isoforms were found in all the plants treated with biocontrol agents while additional PO1, PPO3, PPO4 and PPO5 were observed in biocontrol agents treated plants followed by challenge inoculated with the pathogen.
- Studies on the expression of proteins through SDS PAGE revealed that pretreatment of groundnut plants with biocontrol agents induced ~14 kDa and 25 kDa proteins. Plants treated with biocontrol agents and challenge inoculated with the pathogen had additional proteins of 35, 40, 55 and 97 kDa proteins. Healthy and inoculated plants showed ~14 and 25 kDa protein induction.
- In greenhouse condition, the biocontrol agents *viz.*, Pf1, Pf2, Bs1 and Bs5 as seed treatment and foliar spray and the leaf extract of *A. indicum* and *D. metel* as foliar spray recorded lesser disease intensity. However, Pf1 as seed treatment plus foliar spray was significantly superior than others in reducing the disease incidence.
- The results of the three field trials revealed that seed treatment with Pf1 @ 10 g/kg of seed followed by two foliar spray on 30 and 40 days after sowing @ 0.2% significantly recorded lesser disease incidence with increased yield than other treatments.
- The phyllosphere population of bacterial isolates were found to be higher on 15 days after foliar spray and thereafter the population of *P. fluorescens* and *B. subtilis* get declined.
- The population of the *P. fluorescens (*Pf1) and *B. subtilis* (BS1) were higher up to 90 days in the talc based formulation (shelf life period) and thereafter declined. Hence, the talc based formulation can be stored upto 90 days without affecting the population.

- Through ELISA, *A. alternata* can be detected as early as 4th day after inoculation i.e., 6 days before the symptom appearance in the inoculated plants.
- In the epidemiological studies in the first season trial rabi 2002-03, in respect of first sowing, there was positive correlation for maximum temperature, minimum temperature and GDD with the Alternaria leaf blight disease incidence. In the second sowing, minimum temperature, GDD and RH (e) were significantly associated with the disease incidence.
- In kharif 2003-04, the correlation study indicated that there was a positive correlation of DV for first sowing and morning RH for the second sowing. Interestingly all the weather parameters had no association with the disease incidence during third sowing.
- In Rabi, 2003-´04, minimum temperature and GDD had positive correlation with the disease incidence in all the three sowings. In addition, maximum temperature and bright sunshine hours had positive correlation with disease for first sowing while the maximum temperature and evening relative humidity have contributed for the disease incidence respectively for the second and third sowing.

Table 1. Incidence of Alternaria leaf blight in major groundnut growing areas of Tamil Nadu

Districts / Location	*Per cent Disease Index
Coimbatore (Aliyar)	60.12[ab]
Cuddalore (Valayamadevi)	63.22 [a]
Dharmapuri (Thimapuram)	42.55[de]
Erode (Kalvettupalayam)	46.66[cd]
Madurai (Usilampatti)	59.35[ab]
Salem (Omalur)	62.27[ab]
Pudukkottai (Alangudi)	57.83[b]
Thiruvannamalai (Velavaram)	38.33[ef]
Trichy (Vaiyalur)	33.77[f]
Vilupuram (Pichanatham)	51.33[c]

.

Table 2. Effect of strains of *Pseudomonas fluorescens* on radial mycelial growth of *A. alternata*

Strains	*Mycelial growth (mm)	Per cent reduction over control	Inhibition zone (mm)
Pf1	43.1[a]	51.18	16.20 [a]
Pf2	48.1[b]	45.52	15.30 [a]
Pf3	47.2[b]	46.54	11.20 [b]
Pf4	52.1[c]	40.96	7.10 [c]
Pf5	55.2[c]	37.48	5.10[d]
Control	88.3[d]	-	-

*Values are mean of three replications.

In a column, means followed by a common letter are not significantly different at 5% level by DMRT

Table 3. Effect of *Bacillus* strains on the mycelial growth of *A. alternata*

Sl. No	**Treatments**	***Mycelial growth of the pathogen (mm)**	**Per cent reduction over control**	**Inhibition zone (mm)**
1	Bs1	41.3^{a}	53.33^{a}	21.0^{a}
2	Bs2	45.2^{bc}	49.92^{cd}	12.2^{dc}
3	Bs3	49.1^{e}	44.51^{f}	11.2^{e}
4	Bs4	44.2^{bc}	50.05^{bc}	13.3^{cd}
5	Bs5	43.3^{ab}	51.07^{b}	19.3^{b}
6	Bs6	45.2^{bc}	48.92^{cd}	$15.2b^{c}$
7	Bs7	47.3^{cd}	46.55^{e}	10.5^{e}
8	Bs8	50.2^{e}	43.27^{f}	16.4^{bc}
9	Bs9	45.3^{bc}	48.81^{cd}	17.3^{b}
10	Bs10	45.5^{bc}	48.92^{cd}	9.1^{ef}
11	Control	88.5^{f}	-	-

***Values are means of three replications**

In a column, means followed by a common letter are not significantly different at 5% level by DMRT

Table 4. Effect of plant products on the mycelial growth of *A. alternata*

Sl. No	Treatments	*Mycelial growth of the pathogen (mm)	Per cent reduction over control
1	***A. indicum***	21.20[a]	75.58
2	***A. indica***	44.10[d]	48.83
3	*B. spectablis*	55.20[f]	36.04
4	*C. roseuss*	54.20[f]	37.20
5	*Acalypha indica*	70.10[g]	18.60
6	*Ocimum sanctums*	51.10[e]	40.69
7	*L.inermis*	40.20[c]	53.48
8	*D. metel*	25.20[b]	70.92
9	*Prosopis juliflora*	61.20[h]	29.06
10	*Tridox procumbens*	63.30[h]	26.74
11	*Cyprus rotandus*	68.10[j]	20.93
12	*Vitex negunda*	71.20[k]	17.44
13	*Ipomea carnea*	78.10[n]	0.9.3
14	*Adathoda vessica*	71.20[k]	17.44
15	*Agave americana*	73.20[lm]	15.11
16	*Neerium indicum*	65.20[i]	24.41
17	Control	86.50[o]	-

*Values are means of three replications

In a column, means followed by a common letter are not significantly different at 5% level by DMRT

Table 5. Plant growth promoting activity of various antagonistic bacteria

Sl. No	Treatments	*15 Days After Sowing (DAS)			
		Germination percentage	**Root length (cm)**	**Shoot length (cm)**	**Vigor index**
1.	**Pf1**	76.00[a] (60.68)#	11.30 [a]	17.90 [a]	2212.92 [a]
2.	Pf2	70.70[b] (57.24)	10.50 [ab]	17.90 [a]	2007.88 [b]
3.	Bs1	70.70 [b] (57.23)	10.30 [ab]	16.50 [b]	2068.57 [b]
4.	Bs5	66.00 [c] (54.34)	8.40 [ab]	14.90 [c]	1537.80 [c]
5.	Control	56.00 [d] (48.45)	7.40 [b]	14.60 [d]	1232.00 [d]

*Values are mean of three replications.

In a column, means followed by a common letter are not significantly different at 5% level by DMRT

#Figures in the parentheses are arcsine transformed values

Table 6. IAA, Salicylic acid and Siderophore production by antagonistic bacteria

Sl. No.	Treatments	***Indole acetic acid (IAA) (µg/ml)**	***Salicylic acid (SA) (µg/ml)**	***Siderophore production (µ mol Benzoic acid /ml)**
1.	**Pf1**	54.70 [a]	28.10 [a]	9.30 [a]
2.	Pf2	48.30 [b]	26.80 [b]	8.80 [a]
3.	Pf3	34.70 [c]	15.40 [c]	5.80 [b]
4.	**Pf4**	34.51[c]	13.50 [d]	5.50 [b]
5.	Pf5	30.10 [d]	13.12	5.38 [b]

*Values are mean of three replications.

In a column, means followed by a common letter are not significantly different at 5% level by DMRT

Table 7. Hydrogen cyanide production by antagonistic bacteria

Sl. No	Treatments	***HCN production**	***HCN production (1 unit= 0.001 Absorbance at 625 nm)**
1.	**Pf1**	++	0.080 [a]
2.	Pf2	++	0.070 [a]
3.	Pf3	+	0.057 [b]
4.	**Pf4**	+	0.052 [b]
5.	Pf5	+	0.050 [bc]

*Values are mean of three replications.

In a column, means followed by a common letter are not significantly different at 5% level by DMRT

Table 8. Phenol and protein content of plant products

Sl. No	Plant products (10%)	*Phenol content	**Soluble protein content
1	*A. indicum*	242.47[a]	1.421[a]
2	*D. metel*	234.39[b]	1.337[ab]
3	*L. inermis*	184.21[c]	1.233[c]
4	*A. indica*	173.41[d]	1.131[cd]

* µg of catechol/g of leaf tissue

** µg/g of fresh leaf tissue

Values are mean of three replications.

In a column, means followed by a common letter are not significantly different at 5% level by DMRT

Table 9. Thin Layer Chromatographic separation of secondary metabolites from the plant products

Sl. No	Plant products	Rf value	**Antimicrobial activity
A. Phenolic compounds[1]			
1	**A. indicum**	+(0.745)	4.90
2	*D. metel*	+ (0.518)	4.80
3	*L. inermis*	+ (0.482)	-
4	*A. indica*	+ (0.360)	-
B. Alkaloid compounds[2]			
1	*A. indicum*	+ (0.880)	6.80
2	*D. metel*	+ (0.972)	5.20
3	*L. inermis*	-	-
4	*A. indica*	-	-

** Inhibition zone (mm)
Values are mean of three replications.

1.Solvent system: chloroform : methanol (90: 10)
2.Solvent system: chloroform : methanol (15:5)

Table 10. Induction of phenylalanine ammonia lyase activity in groundnut plants in response to treatment with biocontrol agents and plant products

Sl. No	Treatments	*n mol of trans cinnamic acid/ mg				
		Days after treatment				
		1	**3**	**5**	**7**	**Mean**
1.	ST+FS (Pf1)	120.00 ^{ab}	133.03 ^{bcd}	157.03 ^{cd}	142.00 ^{cde}	138.01
2.	ST+FS (Pf1+PI)	123.03 ^{ab}	158.03 ^{a}	192.00 ^{a}	187.96 ^{a}	165.25
3.	ST+FS (Pf2)	119.00 ^{ab}	129.00 ^{bcd}	148.00 ^{de}	137.03 $^{c-f}$	133.25
4.	ST+FS (Pf2+PI)	122.03 ^{ab}	142.00 ^{ab}	180.00 ^{ab}	170.00 ^{b}	153.50
5.	ST+FS (Bs1)	131.03 ^{a}	134.60 ^{bcd}	147.33 ^{de}	131.90 ^{def}	136.21
6.	ST+FS (Bs1+PI)	120.00 ^{ab}	140.00 ^{b}	167.03 ^{bc}	152.00 ^{c}	144.75
7.	ST+FS (Bs5)	115.03 ^{ab}	120.00 ^{cd}	135.03 ^{ef}	127.96 ^{efg}	124.50
8.	ST+FS (Bs5+PI)	119.00 ^{ab}	130.00 ^{bcd}	158.00 ^{cd}	149.03 ^{cd}	139.00
9.	*A. indicum*	112.03 ^{b}	118.00 ^{cd}	134.00 ^{ef}	124.00 ^{efg}	122.00
10.	*A. indicum*+PI	131.03 ^{a}	135.66 ^{bc}	146.26 ^{de}	149.33 ^{cd}	140.57
11.	*D. metel*	111.03 ^{b}	116.00 ^{d}	124.00 ^{f}	112.00 ^{gh}	115.75
12.	*D. metel* +PI	115.03 ^{ab}	120.00 ^{cd}	130.00 ^{ef}	120.00 ^{fg}	121.25
13.	Inoculated control	92.00 ^{c}	97.00 ^{e}	98.96 ^{g}	96.00 ^{h}i	95.99
14.	Healthy	80.00 ^{c}	83.00 ^{e}	88.03 ^{g}	85.03 i	84.01
Mean		107.55	125.45	143.26	134.40	129.57

*Values are mean of three replications.
PI- Pathogen inoculation, ST- Seed treatment (10g/kg), FS- Foliar spray

Factors	SED	CD (P=0.05)
Days	0.45	1.102**
Treatments	1.06	2.109**
Days x Treatments	2.12	4.2**

Table 11. Induction of peroxidase activity in groundnut plants in response to treatment with biocontrol agents and plant products

Sl. No	Treatments	*Changes in absorbance/min/mg (1 unit = 0.001 absorbance)				
		Days after treatment				
		1	3	5	7	Mean
1.	ST+FS (Pf1)	174.00 [a]	189.03 [cd]	231.03 [d]	187.00 [d]	195.26
2.	ST+FS (Pf1+PI)	180.00 [a]	284.00 [a]	374.00 [a]	297.96 [a]	283.99
3.	ST+FS (Pf2)	170.00 [a]	180.00 [de]	212.00 [de]	178.00 [d]	185.00
4.	ST+FS (Pf2+PI)	177.96 [a]	278.00 [a]	313.00 [b]	259.00 [b]	256.99
5.	ST+FS (Bs1)	188.16 [a]	188.66 [cd]	203.53 [e]f	182.03 [d]	190.60
6.	ST+FS (Bs1+PI)	174.00 [a]	156.00 [e]	280.00 [c]	233.00 [bc]	210.75
7.	ST+FS (Bs5)	163.03 [a]	170.00 [de]	188.00 [ef]	153.00 [e]	168.50
8.	ST+FS (Bs5+PI)	170.00 [a]	212.00 [bc]	273.00 [c]	227.03 [c]	220.50
9.	*A. indicum*	162.03 [a]	167.03 [de]	185.03 [f]	151.03 [e]	166.28
10.	*A. indicum*+PI	188.16 [a]	222.60 [b]	286.20 [c]	245.26 [bc]	235.55
11.	*D. metel*	160.00 [a]	165.03 [de]	183.00 [f]	150.00 [e]	164.50
12.	*D. metel* +PI	166.00 [a]	208.03 [bc]	268.00 [c]	222.03 [c]	216.01
13.	Inoculated control	110.00 [b]	128.00 [f]	148.00 [g]	123.00 [f]	127.25
14.	Healthy	84.00 [c]	87.03 [g]	91.00 [h]	88.00 [g]	87.50
Mean		151.56	188.24	231.13	192.60	193.48

*Values are mean of three replications.
PI- Pathogen inoculation, ST- Seed treatment (10g/kg), FS- Foliar spray

Factors	SED	CD (P=0.05)
Days	2.81	5.846**
Treatments	6.23	12.35**
Days x Treatments	12.46	24.70**

Table 12. Induction of polyphenol oxidase activity in groundnut plants in response to treatment with biocontrol agents and plant products

Sl. No	Treatments	*Changes in absorbance/min/mg (1 unit = 0.001 absorbance)				
		Days after treatment				
		1	3	5	7	Mean
1.	ST+FS (Pf1)	12.23 ^{bcd}	14.30 ^{cd}	16.20 ^{ef}	14.13 ^{def}	14.21
2.	ST+FS (Pf1+PI)	14.23 ^{a}	18.23 ^{a}	23.40 ^{a}	20.10 ^{a}	18.99
3.	ST+FS (Pf2)	11.80 ^{bcd}	13.50^{d}	15.10 ^{fg}	13.80 $^{d-g}$	13.55
4.	ST+FS (Pf2+PI)	13.30 ^{ab}	16.50 ^{b}	21.30 ^{b}	19.30 ^{a}	17.60
5.	ST+FS (Bs1)	12.56 $^{a-d}$	13.60 ^{d}	15.13 ^{fg}	13.16 ^{efg}	13.61
6.	ST+FS (Bs1+PI)	13.00 ^{abc}	15.80 ^{bc}	19.70 ^{bc}	17.20 ^{b}	16.42
7.	ST+FS (Bs5)	11.20 ^{cde}	11.76 ^{e}	13.50 ^{gh}	12.33 ^{fgh}	12.20
8.	ST+FS (Bs5+PI)	12.30 $^{a-d}$	14.23 ^{cd}	17.80 ^{de}	15.13 ^{cd}	14.86
9.	*A. indicum*	11.10 ^{cde}	11.66 ^{e}	13.30 ^{ghi}	12.10 ^{gh}	12.04
10.	*A. indicum*+PI	13.53 ^{ab}	14.86 ^{bcd}	18.60 ^{cd}	16.26 ^{bc}	15.81
11.	*D. metel*	11.033 ^{cde}	11.50 ^{e}	13.00 ^{hi}	12.00 ^{gh}	11.88
12.	*D. metel* +PI	12.00 ^{bcd}	13.90 ^{cd}	17.00^{de}	14.46 ^{cde}	14.34
13.	Inoculated control	10.80 ^{de}	11.10 ^{e}	11.50 ^{ij}	11.20 ^{hi}	11.15
14.	Healthy	9.50 ^{e}	9.90 ^{e}	10.20 ^{j}	9.80 ^{i}	9.85
Mean		11.59	13.63	16.58	14.39	14.04

*Values are mean of three replications.

PI- Pathogen inoculation, ST- Seed treatment (10g/kg), FS- Foliar spray

Factors	SED	CD (P=0.05)
Days	0.195	0.477**
Treatments	0.44	0.864**
Days x Treatments	0.87	1.728**

Table 13. Induction of chitinase activity in groundnut plants in response to treatment with biocontrol agents and plant products

Sl. No	Treatments	*n mol of GluNac/min/mg				
		Days after treatment				
		1	3	5	7	Mean
1.	ST+FS (Pf1)	6.00 ^{ab}	10.10 ^{c}	12.70 ^{bc}	11.13 ^{abc}	9.98
2.	ST+FS (Pf1+PI)	6.80 ^{a}	12.80 ^{a}	16.20 ^{a}	12.20 ^{a}	12.00
3.	ST+FS (Pf2)	4.30 ^{d}	7.40 ^{d}	12.20 ^{cd}	9.90 ^{def}	8.45
4.	ST+FS (Pf2+PI)	5.60 ^{bc}	11.30 ^{b}	13.40 ^{b}	10.00 $^{c-f}$	10.07
5.	ST+FS (Bs1)	4.70 ^{cd}	5.40 ^{e}	12.19 ^{cd}	10.357 $^{c-f}$	8.16
6.	ST+FS (Bs1+PI)	5.10 ^{bcd}	10.20 ^{c}	13.20 ^{bc}	10.00 $^{c-f}$	9.62
7.	ST+FS (Bs5)	4.10 ^{d}	4.70 ^{e}	11.10 ^{de}	10.70 $^{b-e}$	7.65
8.	ST+FS (Bs5+PI)	4.90 ^{bcd}	9.90 ^{c}	13.10 ^{bc}	11.10 $^{a-d}$	9.75
9.	*A. indicum*	4.10 d	4.70 ^{e}	10.85 ^{e}	9.800 ^{ef}	7.36
10.	*A. indicum*+PI	4.70 ^{cd}	10.07 ^{c}	13.54 ^{b}	11.81 ^{ab}	10.03
11.	*D. metel*	4.10 ^{d}	4.70 ^{e}	10.25 ^{e}	9.300 ^{f}	7.09
12.	*D. metel* +PI	4.20 ^{d}	9.30 ^{c}	12.11 ^{cd}	10.22 $^{c-f}$	8.95
13.	Inoculated control	4.00 ^{d}	4.50 ^{e}	8.20 f	7.200 ^{g}	5.97
14.	Healthy	4.00 ^{d}	4.30 ^{e}	6.30 ^{g}	5.700 ^{h}	5.07
Mean		4.71	7.81	11.81	9.63	8.58

*Values are mean of three replications.
PI- Pathogen inoculation, ST- Seed treatment (10g/kg), FS- Foliar spray

Factors	SED	CD (P=0.05)
Days	0.229	0.560**
Treatments	0.267	0.530**
Days x Treatments	0.537	1.066**

Table 14. Induction of β1,3-glucanase activity in groundnut plants in response to treatment with biocontrol agents and plant products

Sl. No	Treatments	*μg of glucanase released /mg				
		Days after treatment				
		1	3	5	7	Mean
1.	ST+FS (Pf1)	35.03 ^{ab}	38.00 ^{d}	41.03 ^{cd}	29.00 ^{ef}	35.76
2.	ST+FS (Pf1+PI)	37.00 ^{a}	58.03 ^{a}	68.00 ^{a}	52.03 ^{a}	53.76
3.	ST+FS (Pf2)	29.00 ^{cd}	31.00 ^{fg}	38.96 ^{def}	33.00 ^{de}	32.99
4.	ST+FS (Pf2+PI)	31.00 ^{bc}	55.03 ^{ab}	61.00 ^{b}	49.00 ^{ab}	49.00
5.	ST+FS (Bs1)	22.40^{fg}	31.80 ^{ef}	41.33 ^{cd}	31.60 ^{de}	31.78
6.	ST+FS (Bs1+PI)	23.00 ^{fg}	53.03 ^{b}	59.00 ^{b}	48.00 ^{ab}	45.75
7.	ST+FS (Bs5)	23.03 ^{fg}	29.00 ^{fgh}	37.00 ^{def}	25.03 ^{fg}	28.51
8.	ST+FS (Bs5+PI)	26.00 ^{def}	45.96 ^{c}	57.00 ^{b}	45.03 ^{bc}	43.50
9.	*A. indicum*	22.03 ^{fg}	28.00 $^{f-i}$	36.00 ^{ef}	24.00 ^{gh}	27.50
10.	*A. indicum*+PI	28.03 ^{cde}	40.26 ^{d}	44.53 ^{c}	42.50 ^{c}	38.83
11.	*D. metel*	21.03 ^{gh}	27.03 ^{ghi}	35.03 ^{f}	23.00 ^{gh}	26.52
12.	*D. metel* +PI	24.00 ^{efg}	36.00 ^{de}	40.00 ^{cde}	35.03 ^{d}	33.75
13.	Inoculated control	20.00 ^{gh}	25.96 ^{hi}	28.96 ^{g}	20.00 ^{hi}	23.73
14.	Healthy	17.00 ^{h}	24.00 ^{i}	27.00 ^{g}	17.00 ^{i}	21.25
Mean		24.24	38.08	43.92	33.87	35.19

*Values are mean of three replications.
PI- Pathogen inoculation, ST- Seed treatment (10g/kg), FS- Foliar spray

Factors	**SED**	**CD (P=0.05)**
Days	0.45	1.1028**
Treatments	2.72	5.109**
Days x Treatments	2.81	5.2**

Table 15. Induction of TAL activity in groundnut plants in response to treatment with biocontrol agents and plant products

Sl. No	Treatments	*Changes in absorbance/min/mg				
		Days after treatment				
		1	**3**	**5**	**7**	Mean
1.	ST+FS (Pf1)	16.03 ^{ab}	18.30 ^{bc}	18.43 ^{cd}	15.86 ^{bc}	17.15
2.	ST+FS (Pf1+PI)	16.20 ^{ab}	20.70 ^{a}	23.13^{a}	19.23 ^{a}	19.81
3.	ST+FS (Pf2)	16.13 ^{ab}	17.70 ^{bc}	18.13 ^{cde}	16.13 ^{bc}	17.02
4.	ST+FS (Pf2+PI)	16.46 ^{ab}	18.73 ^{ab}	21.26 ^{ab}	18.00 ^{ab}	18.61
5.	ST+FS (Bs1)	17.90 ^{a}	18.20 ^{bc}	18.03 ^{cde}	16.26 ^{bc}	17.60
6.	ST+FS (Bs1+PI)	15.90 ^{ab}	18.23 ^{bc}	20.76 ^{b}	17.76 ^{ab}	18.16
7.	ST+FS (Bs5)	15.56 ^{ab}	16.10 ^{cd}	16.20 ^{def}	14.26 ^{cd}	15.53
8.	ST+FS (Bs5+PI)	15.63 ^{ab}	17.20 ^{bc}	19.23 ^{bc}	16.26 ^{bc}	17.08
9.	*A. indicum*	15.43 ^{ab}	16.00 ^{cd}	15.96 ^{ef}	14.23 ^{cd}	15.40
10.	*A. indicum*+PI	17.36 ^{ab}	18.00 ^{bc}	20.13 ^{bc}	17.43 ^{ab}	18.23
11.	*D. metel*	15.33 ^{b}	16.00 ^{cd}	17.76 ^{cde}	14.13 ^{cd}	15.80
12.	*D. metel* +PI	15.46 ^{ab}	16.80 ^{bc}	18.96 ^{bc}	15.93 ^{bc}	16.79
13.	Inoculated control	11.23 ^{c}	13.93 ^{d}	14.23 ^{fg}	12.23 ^{de}	12.90
14.	Healthy	9.60 ^{c}	11.03 ^{e}	12.76 ^{g}	11.03 ^{e}	11.10
Mean		14.81	16.92	18.21	15.62	16.51

*Values are mean of three replications.
PI- Pathogen inoculation, ST- Seed treatment (10g/kg), FS- Foliar spray

Factors	**SED**	**CD (P=0.05)**
Days	0.255	0.622**
Treatments	0.527	1.046**
Days x Treatments	1.055	2.093**

Table 16. Phenolic content in groundnut plants treatment with biocontrol agents and plant products

Sl. No	Treatments	*µg /mg of fresh weight				
		Days after treatment				
		1	3	5	7	Mean
1.	ST+FS (Pf1)	118.20 [a]	121.23 [bc]	131.20 [b]	127.23 [d]	124.46
2.	ST+FS (Pf1+PI)	119.23 [a]	174.33 [a]	224.43 [a]	197.30 [b]	178.82
3.	ST+FS (Pf2)	116.20 [a]	119.96 [bc]	128.96 [b]	115.23 [de]	120.09
4.	ST+FS (Pf2+PI)	118.70 [a]	158.73 [a]	222.26 [a]	219.16 [a]	179.71
5.	ST+FS (Bs1)	132.66 [a]	127.53 [bc]	134.73 [b]	126.66 [d]	130.40
6.	ST+FS (Bs1+PI)	117.13 [a]	138.93 [b]	219.90 [a]	197.23 [b]	168.30
7.	ST+FS (Bs5)	117.46 [a]	121.20 [bc]	125.26 [b]	113.30 [de]	119.30
8.	ST+FS (Bs5+PI)	117.20 [a]	129.00 [bc]	218.13 [a]	179.23 [bc]	160.89
9.	*A. indicum*	116.00 [a]	120.40 [bc]	124.03 [bc]	112.40 [de]	118.20
10.	*A. indicum*+PI	130.00 [a]	135.86 [bc]	230.60 [a]	194.10 [bc]	172.64
11.	*D. metel*	115.06 [a]	119.50 [bc]	123.36 [bc]	112.03 [de]	117.49
12.	*D. metel* +PI	115.06 [a]	127.83 [bc]	215.03 [a]	177.06 [c]	158.75
13.	Inoculated control	111.80 [a]	115.96 [c]	117.23 [bc]	101.23 [e]	111.55
14.	Healthy	93.53 [b]	98.06 [d]	105.03 [c]	101.50 [e]	99.53
Mean		109.35	129.18	165.73	148.12	140.01

*Values are mean of three replications.
PI- Pathogen Inoculation, ST- Seed treatment (10g/kg), FS- Foliar spray

Factors	**SED**	**CD (P=0.05)**
Days	2.016	4.92**
Treatments	4.48	8.88**
Days x Treatments	7.28	17.76**

Table 17. Protein content in groundnut plants treatment with biocontrol agents and plant products

Sl. No	Treatments	*µg /mg of fresh weight				
		Days after treatment				
		1	3	5	7	Mean
1.	ST+FS (Pf1)	0.27 ab	0.57 a	0.62 e	0.51 b	0.49
2.	ST+FS (Pf1+PI)	0.29 a	0.59 a	0.93 a	0.62 a	0.60
3.	ST+FS (Pf2)	0.24 ab	0.45 c	0.59 e	0.32 f	0.40
4.	ST+FS (Pf2+PI)	0.25 ab	0.55 ab	0.82 b	0.61 a	0.56
5.	ST+FS (Bs1)	0.23 b	0.42 cd	0.60 e	0.38 e	0.41
6.	ST+FS (Bs1+PI)	0.23 b	0.51 b	0.77 bc	0.58 a	0.52
7.	ST+FS (Bs5)	0.23 b	0.32 fgh	0.50 f	0.32 f	0.34
8.	ST+FS (Bs5+PI)	0.22 b	0.37 ef	0.72 d	0.44 cd	0.43
9.	*A. indicum*	0.24 ab	0.30 ghi	0.45 g	0.33 f	0.33
10.	*A. indicum*+PI	0.25 ab	0.38 de	0.73 cd	0.48 bc	0.46
11.	*D. metel*	0.23 b	0.29 h[i]	0.41 g	0.33 f	0.31
12.	*D. metel* +PI	0.22 b	0.35 efg	0.64 e	0.42 de	0.40
13.	Inoculated control	0.22 b	0.28 h[i]	0.33 h	0.28 fg	0.27
14.	Healthy	0.21 b	0.25 i	0.26 i	0.24 g	0.24
Mean		0.289	0.402	0.598	0.419	0.411

*Values are mean of three replications.
PI- Pathogen Inoculation, ST- Seed treatment (10g/kg), FS.- Foliar spray

Factors	**SED**	**CD (P=0.05)**
Days	0.006	0.014**
Treatments	0.012	0.024**
Days x Treatments	0.024	0.048**

Table18. Effect of biocontrol agent and plant products against leaf blight, under glass house condition

Sl.No	Treatments (%)	*Per cent disease index (PDI)	Per cent reduction ov control
1.	Pf1 (ST)	49.23^{ghi} (44.56)	14.03
2.	Pf1 (ST+FS)	24.17^{b} (29.44)	57.79
3.	Pf1 (FS)	32.23^{d} (34.59)	43.72
4.	Pf2 (ST)	49.27^{ghi} (44.59)	13.96
5.	Pf2 (ST+FS)	26.21^{bc} (30.79)	54.23
6.	Pf2 (FS)	34.43^{de} (35.93)	39.88
7.	Bs1 (ST)	50.13^{hi} (45.08)	12.46
8.	Bs1 (ST+FS)	27.13^{c} (31.39)	52.62
9.	Bs1 (FS)	35.11^{de} (36.34)	38.69
10.	Bs5 (ST)	50.93^{I} (45.54)	11.07
11.	Bs5 (ST+FS)	28.21^{c} (32.08)	50.74
12.	Bs5 (FS)	36.22^{e} (36.99)	36.75
13.	**A. indicum *(FS)***	44.93^{f} (42.09)	45.32
14.	*D. metel* (FS)	47.23^{fgh} (43.42)	41.80
15.	Mancozeb (FS)	21.32^{a} (27.49)	62.77
16.	**Control**	57.27^{j} (49.19)	-

ST - Seed Treatment (10g/kg), FS - Foliar Spray

*Values are mean of three replications.

In column, means followed by a common letter are not significantly different at 5% level by DMRT
Figures in the parentheses are arcsine transformed value

Table 19. Effect of biocontrol agents and plant products on biometric observations on groundnut plants, under glass house condition

Sl.No	Treatments	*Plant Height (cm)			*No. of leaflets		
		30	45	70	30	45	70
1.	Pf1 (ST)	7.07 $^{b-e}$	8.11^{de}	11.92^{ghi}	4.11^{a-d}	6.12^{bc}	10.83 $^{d-g}$
2.	Pf1 (ST+FS)	9.78^{a}	13.89^{b}	21.23^{ab}	6.13^{a}	8.28^{d}	16.8^{1k}
3.	Pf1 (FS)	8.31^{abc}	9.11^{cde}	17.12^{cde}	4.12^{a-d}	5.07^{b}	12.23^{e-i}
4.	Pf2 (ST)	6.21^{de}	10.21^{b-e}	11.12^{hi}	4.14^{a-d}	4.08^{a}	8.29^{ab}
5.	Pf2 (ST+FS)	6.23^{cde}	12.33^{bc}	20.21^{abc}	5.07^{abc}	8.04^{d}	14.38^{ij}
6.	Pf2 (FS)	8.98^{ab}	9.11^{cde}	15.11^{d-g}	4.11^{a-d}	5.11^{ab}	11.00^{d-h}
7.	Bs1 (ST)	7.13^{b-ed}	8.22^{de}	12.17^{ghi}	3.03^{d}	5.12^{ab}	10.11^{b-e}
8.	Bs1 (ST+FS)	8.23^{a-d}	15.47^{def}	18.27^{bcd}	5.21^{ab}	8.17^{d}	13.01^{g-j}
9.	Bs1 (FS)	8.83^{ab}	9.21^{cde}	14.11^{e-h}	4.33^{a-d}	6.12^{bcd}	9.81^{bcd}
10.	Bs5 (ST)	7.14^{b-e}	8.33^{de}	10.33^{i}	5.17^{ab}	6.13^{bcd}	11.81^{d-h}
11.	Bs5 (ST+FS)	8.23^{a-d}	8.12^{de}	16.67^{cde}	5.14^{ab}	8.21 ^{d}	13.11^{hij}
12.	Bs5 (FS)	9.24^{ab}	9.13^{cde}	14.12^{e-h}	3.21^{cd}	6.22^{bcd}	12.33^{f-i}
13.	**A. indicum *(FS)***	9.11^{ab}	19.24^{a}	14.89^{d-g}	5.21^{ab}	8.17 ^{d}	11.11^{d-h}
14.	*D. metel* (FS)	8.15^{a-d}	10.18^{b-e}	17.38^{cde}	5.08^{abc}	8.12 ^{d}	12.27^{e-i}
15.	Mancozeb (FS)	9.17^{ab}	10.33^{b-e}	23.42^{a}	5.05^{abc}	8.24 ^{d}	15.12^{jk}
16.	**Control**	8.21^{a-d}	10.18^{b-e}	11.11^{bcd}	4.31^{a-d}	7.13^{cd}	10.11^{b-e}

ST - Seed Treatment (10g/kg), FS - Foliar Spray
*Values are mean of three replications.
In column, means followed by a common letter are not significantly different at 5% level by DMRT

Table 20. Effect of biocontrol agents and plant products against Alternaria leaf blight of groundnut (Trial-I Rabi, 2002-'03)

Sl. No	Treatments	*Per cent disease index (PDI)	Per cent reduction over control	*Yield (Kg/ha)
1	Pf1 (ST+FS)	23.23 (28.82) [b]	60.78	1342[b]
2	Pf2 (ST+FS)	28.11 (32.02) [c]	52.54	1319[c]
3	Bs1 (ST+FS)	30.44 (33.49) [d]	48.60	1299[d]
4	Bs5 (ST+FS)	32.34 (34.65) [e]	45.39	1213[e]
5	**A. indicum *(FS)***	33.28 (34.12) [de]	43.81	1178[f]
6	**D. metel *(FS)***	34.10 (35.29) [ef]	42.42	1159 [f]
7	Mancozeb	20.14 (26.65) [a]	65.99	1417[a]
8	Control	59.23 (50.33) [g]	50.32[g]	940[f]

ST - Seed Treatment (10g/kg), FS - Foliar Spray
*Values are mean of three replications.

In a column, means followed by a common letter are not significantly different at 5% level by DMRT
Figures in the parentheses are arcsine transformed values

Table 21. Effect of biocontrol agents on Alternaria leaf blight of groundnut (Trial-II Kharif, 2003-'04)

Sl. No	Treatments	*Per cent disease index (PDI)	*Per cent reduction over control	*Yield (Kg/ha)
1.	Pf1 (ST+FS)	25.47[b](32.84)	58.87	1372 [b]
2.	Pf2 (ST+FS)	28.11[cd](32.02)	54.61	1310 [c]
3.	Bs1 (ST+FS)	29.78[cd](35.59)	51.91	1228 [d]
4.	Bs5 (ST+FS)	31.28[de](36.76)	49.49 [e]	1177 [e]
5.	**A. indicum *(FS***	34.81[f](35.72)	43.79	1110[f]
6.	**D. metel *(FS)***	33.28[f](34.12)	46.26	1020[g]
7.	Mancozeb	19.13[a](26.50)	69.11	1521 [a]
8.	Control	61.93[g](51.55)	-	1012 [h]

ST - Seed Treatment (10g/kg), FS - Foliar Spray
*Values are mean of three replications.
In a column, means followed by a common letter are not significantly different at 5% level by DMRT
Figures in the parentheses are arcsine transformed value

Table 22. Effect of biocontrol agents and plant products on Alternaria leaf blight of groundnut (Trial-III Rabi, 2003-'04)

Sl. No.	Treatments	*Per cent disease index (PDI)	Per cent reduction over control	*Yield (Kg/ha)
1.	Pf1 (ST+FS)	23.24^{b}(28.82)	51.89	1412^{b}
2.	Pf2 (ST+FS)	26.12^{c}(30.73)	45.93	1334^{c}
3.	Bs1 (ST+FS)	27.33^{cd}(31.51)	43.42	1311^{d}
4.	Bs5 (ST+FS)	30.44^{d}(33.49)	36.99	1461^{e}
5.	**A. indicum *(FS)***	31.28^{de}(36.76)	35.25	1212^{f}
6.	**D. metel *(FS)***	32.34^{ef}(34.65)	33.05	1109^{g}
7.	Mancozeb	18.33^{a}(25.34)	62.05	1401^{a}
8.	Control	48.31^{g}(44.03)	-	1033^{h}

ST - Seed Treatment (10g/kg), FS - Foliar Spray
*Values are mean of three replications.
In a column, means followed by a common letter are not significantly different at 5% level by DMRT

Table 23. Survival of *P. fluorescens* and *B. subtilis* on phyllosphere

Days after spray	***Phyllosphere population of *P. fluorescens* and *B. subtilis* (cfu x10^8 /g)**	
	P. fluorescens	***B. subtilis***
0	3.0 [e]	4.0 [e]
5	3.3 [e]	4.2 [e]
10	3.7 [e]	4.7 [e]
15	8.3 [a]	8.1 [a]
30	7.2 [b]	6.9 [b]
45	6.5 [c]	5.1 [c]
60	5.2 [d]	4.8 [d]
75	3.2 [f]	3.9 [f]

*Values are mean of three replications.
In a column, means followed by a common letter are not significantly different (P=0.05) by DMRT

Table 24. Population dynamics of *P. fluorescens* and *B. subtilis* at different days of storage period

Days after storage	*Population of *P. fluorescens* and *B. subtilis* (cfux10^8 /g)	
	P. fluorescens	***B. subtilis***
0	10.8	10.5
30	10.5	10.3
45	9.3	9.7
60	8.7	9.1
90	7.9	7.8
120	5.8	5.2
150	2.5	3.6

*Values are mean of three replications.
In a column, means followed by a common letter are not significantly different at 5% level by DMRT

Table 25. Determination of titre value of the antiserum of *Alternaria alternata*

Antigen dilution	Antiserum dilution							
	1:50	**1:100**	**1:200**	**1:500**	**1:1000**	**1:3000**	**Healthy**	**Buffer control**
1:50	2.321	2.213	2.121	1.983	1.283	0.981	0.531	0.072
1:100	2.212	2.184	2.083	1.897	1.181	1.011	0.611	0.061
1:200	2.131	2.087	2.001	1.739	1.081	1.009	0.531	0.083
1:500	1.921	0.991	0.931	0.921	0.889	0.811	0.489	0.091
1:1000	1.210	0.931	0.821	0.731	0.631	0.521	0.552	0.072
1:3000	0.824	0.131	0.681	0.531	0.421	0.331	0.449	0.063

Table 26. Detection of *Alternaria alternata* using ELISA at different days after inoculation

Antigen dilution	**Antiserum dilution 1:1000**									
	Infected samples (days after inoculation)									
1:100	0	1	2	3	4	5	6	7	8	B
	0.418	0.521	0.693	0.983	1.231	1.421	1.692	1.824	2.032	0.078
Healthy samples (days)										
1:100	0	1	2	3	4	5	6	7	8	B
	0.418	0.423	0.451	0.438	0.512	0.531	0.549	0.561	0.581	0.078

Table 28. Correlation of weather factors with Alternaria leaf blight disease incidence (Rabi, 2002-'03)

Sl. No	Weather parameters	I Sowing	II Sowing	III Sowing
1	Maximum Temperature (^{o}C)	0.7646**	0.5021	0.3774
2	Minimum Temperature (^{o}C)	0.9658**	0.8477**	0.8059**
3	Diurnal Variation (^{o}C)	0.0017	-0.6319	-0.6316
4	Relative Temperature Disparity	-0.4997	-0.7515	-0.7451
5	Growing Degree Days (^{o}C)	0.9052**	0.8172**	0.7188
6	Relative Humidity (m) (%)	0.4493	0.1069	-0.3635
7	Relative Humidity (e) (%)	0.2306	0.6958*	0.7905**
8	Rainfall (Total) (mm)	0.4228	0.1237	-0.0564
9	Bright Sunshine Hour	0.0794	-0.4629	-0.4909

* Significant at 5 %
** Significant at 1 %.

Table 29. Multiple regression between weather factors and Alternaria leaf blight disease incidence (Rabi, 2002-'03)

Sl. No	Weather parameters	I Sowing	II Sowing	III Sowing
1	Constant	963.5564	1992.3770	1992.3770
2	Maximum Temperature (^{o}C)	102.7293	-49.3565	-49.3565
3	Minimum Temperature (^{o}C)	-123.1750	-10.2688	-10.2688
4	Diurnal Variation (^{o}C)	-70.5733	209.2743	209.2743
5	Relative Temperature Disparity	-25.2436	-81.5776	-81.5776
6	Growing Degree Days (^{o}C)	7.9637	-5.4390	-5.4390
7	Relative Humidity (m) (%)	0.7419	3.4523	3.4523
8	Relative Humidity (e) (%)	-2.5398	0.5061	0.5061
9	Rainfall (Total) (mm)	-1.3029	-1.4982	-1.4982
10	Bright Sunshine Hours	-2.8770	4.4046	4.4046
11	R^2 value	0.9931**	0.9940**	0.8894**

* Significant at 5 %
** Significant at 1 %.

Table 30. Regression equation for predicting the Alternaria leaf blight disease incidence with weather factors (Rabi, 2002-'03)

Sl. No	Date of sowing	Prediction equation	R^2 value
1	First sowing (03.01.03)	Y=-52.4149-2.06818 (T max) +5.082203 (T mini) +4.651249 RH (e)	0.9334**
2	Second sowing (15.01.03)	Y=-219.917+9.84599 (T mini) +3.2842 (GDD) -0.104 RH (e)	0.7251**
3	Third sowing (03.02.03)	Y=-112.791+3.122999 (T mini)+3.2512 (GDD)+0.9531 RH (e)	0.6696*

* Significant at 5 %
** Significant at 1 %.

Table 32. Correlation of weather factors with the Alternaria leaf blight disease incidence (Kharif, 2003-'04)

Sl. No	Weather parameters	*I Sowing*	II Sowing	III Sowing
1	Maximum Temperature (^{o}C)	-0.1385	-0.6160	-0.3949
2	Minimum Temperature (^{o}C)	-0.6190	-0.8450	-0.5742
3	Diurnal Variation (^{o}C)	0.6790*	0.3241	-0.0709
4	Relative Temperature Disparity	0.5609	0.5614	0.0103
5	Growing Degree Days (^{o}C)	-0.4069	-0.8171	-0.5187
6	Relative Humidity (m) (%)	0.4958	0.8698**	0.6210
7	Relative Humidity (e) (%)	-0.6730	-0.0796	0.2795
8	Rainfall (Total) (mm)	0.3976	0.4514	0.3889

* Significant at 5 %
** Significant at 1 %.

Table 33. Multiple regression between weather factors and the Alternaria leaf blight disease incidence (Kharif, 2003-'04)

Sl. No	Weather parameters	*I Sowing*	II Sowing	III Sowing
1	Constant	3311.0750	1279.7620	7886.0890
2	Maximum Temperature (^{o}C)	-100.7120	-22.4157	-114.2080
3	Minimum Temperature (^{o}C)	-71.5580	-66.9492	-256.3110
4	Diurnal Variation (^{o}C)	24.6212	-58.1011	62.2895
5	Relative Temperature Disparity	-6.4035	13.4568	-60.3985
6	Growing Degree Days (^{o}C)	163.3035	97.2840	275.2423
7	Relative Humidity (m) (%)	1.0827	2.7177	5.5762
8	Relative Humidity (e) (%)	-1.2081	0.0388	-3.7799
9	Rainfall (Total) (mm)	-2.4981	-2.1487	-8.6912
10	R^2 value	0.9802**	0.8429**	0.9750**

* Significant at 5 %
** Significant at 1 %.

Table 34. Regression equation for predicting the Alternaria leaf blight disease incidence with weather factors (Kharif, 2003-´04)

Sl. No	Date of sowing	Prediction equation	R^2 value
1	First sowing (30.06.03)	Y=-105.869+13.0495 (DV)	0.6868*
2	Second sowing (13.07.03)	Y=-166.257+2.3060 RH (M)	0.8244**

* Significant at 5 %
** Significant at 1 %.

Table 36. Correlation of weather factors with the Alternaria leaf blight disease incidence (Rabi, 2003-04)

Sl. No	Weather parameters	*I Sowing*	II Sowing	III Sowing
1	Maximum Temperature (^{o}C)	0.9400**	0.9058**	0.5654
2	Minimum Temperature (^{o}C)	0.8031**	0.6472*	0.8912**
3	Diurnal Variation (^{o}C)	-0.2866	0.2934	-0.6691
4	Relative Temperature Disparity	-0.5697	-0.0597	-0.8441
5	Growing Degree Days (^{o}C)	0.9055**	0.8599**	0.8091**
6	Relative Humidity (m) (%)	-0.5150	-0.0927	-0.0853
7	Relative Humidity (e) (%)	-0.8418	0.0967	0.8265**
8	Rainfall (Total) (mm)	-0.2775	0.5169	0.5351
9	Bright Sunshine Hours	0.6573*	-0.4583	-0.5633

* Significant at 5 %
** Significant at 1 %

Table 37. Multiple regression analysis between weather factors and the Alternaria leaf blight disease incidence (Rabi, 2003-´04)

Sl. No	Weather parameters	I Sowing	II Sowing	III Sowing
1	Constant (^{o}C)	334.639	-663.93	-81.893
2	Maximum Temperature (^{o}C)	-44.013	-20.073	2.8225
3	Minimum Temperature (^{o}C)	14.0041	15.8357	-8.0207
4	Diurnal Variation (^{o}C)	0.3281	-70.278	-3.5326
5	Relative Temperature Disparity	7.5677	36.6598	-1.5207
6	Growing Degree Days (^{o}C)	42.4266	53.0992	10.5196
7	Relative Humidity (m) (%)	0.8581	-0.6754	1.7325
8	Relative Humidity (e) (%)	1.0166	1.0846	0.6438
9	Rainfall (Total) (mm)	-30.367	2.3843	-0.3764
10	Bright Sunshine Hours	11.163	7.8324	-0.9981
11	R^2 value	0.9884**	0.9842**	0.95698**

* Significant at 5 %
** Significant at 1 %.

Table 38. Regression equation for predicting the Alternaria leaf blight disease incidence with weather factors (Rabi, 2003-'04)

Sl. No	Date of sowing	Prediction equation	R^2 value
1	First sowing (15.12.03)	Y= - 578.81+16.8763 (T max) +10.6009 (T min) -19.176 (GDD)	0.8947**
2	Second sowing (30.12.03)	Y= 240.594-6.3329 (T max)-13.013 (T min)+28.4675 (GDD)	0.8305**
3	Third sowing (14.01.04)	Y= -116.87+5.24851 (T min)-0.6181 (GDD)+0.82834 RH (e)	0.8877**

* Significant at 5 %
** Significant at 1 %.

Table 27. Occurrence of Alternaria leaf blight during Rabi 2002-'03

Sl. No.	I Sowing		II Sowing		III Sowing	
	D/S : 03.01.03		D/S : 19.01.03		D/S : 03.02.03	
	Date of Observation	PDI	Date of observation	PDI	Date of observation	PDI
1	06.02.03	11.33	20.02.03	12.11	05.03.03	13.21
2	13.02.03	13.85	27.02.03	13.50	12.03.03	15.81
3	20.02.03	20.17	06.03.03	17.18	19.03.03	26.10
4	27.02.03	25.24	13.03.03	25.13	26.03.03	32.77
5	06.03.03	28.00	20.03.03	29.11	02.04.03	34.55
6	13.03.03	37.52	27.03.03	32.33	09.04.03	37.62
7	20.03.03	38.19	03.04.03	37.92	16.04.03	40.17
8	27.03.03	40.75	10.03.03	44.25	23.04.03	42.17
9	02.04.03	45.07	18.04.03	46.17	30.04.03	44.47
10	09.04.03	51.24	25.04.03	49.39	07.05.03	48.59
11	16.04.03	54.21	02.05.03	53.39	14.05.03	52.57

D/S – Date of sowing

Table 31. Occurrence of Alternaria leaf blight during Kharif, 2003-‘04

Sl. No.	Kharif season					
	I Sowing		II Sowing		III Sowing	
	D/S : 30.06.03		D/S : 13.07.03		D/S : 28.07.03	
	Date of Observation	PDI	Date of observation	PDI	Date of observation	PDI
1	03.08.03	12.25	17.08.03	11.22	30.08.03	11.88
2	10.08.03	15.43	25.08.03	15.55	07.09.03	18.81
3	17.08.03	21.47	01.09.03	19.75	14.09.03	23.25
4	24.08.03	29.23	08.09.03	24.57	21.09.03	27.65
5	31.08.03	36.89	15.09.03	27.33	28.09.03	29.32
6	07.09.03	41.23	22.09.03	32.71	05.10.03	37.28
7	14.09.03	44.27	29.09.03	36.65	12.10.03	39.21
8	21.09.03	45.62	06.10.03	41.25	19.10.03	47.91
9	28.09.03	47.31	13.10.03	45.57	26.10.03	50.55
10	05.10.03	48.31	20.10.03	49.26	02.11.03	53.45
11	18.10.03	51.27	27.10.03	51.44	09.11.03	54.47

D/S – Date of sowing

Table 35. Occurrence of Alternaria leaf blight during Rabi 2002-'03

Sl. No.	I Sowing		II Sowing		III Sowing	
	D/S : 15.12.03		D/S : 30.12.03		D/S : 14.01.04	
	Date of Observation	PDI	Date of observation	PDI	Date of observation	PDI
1	06.01.04	11.99	10.02.04	12.21	21.02.04	11.88
2	13.01.04	14.44	17.02.04	15.88	28.02.04	13.21
3	20.01.04	18.59	24.02.04	16.25	06.03.04	17.55
4	27.01.04	26.35	02.03.04	21.33	13.03.04	22.47
5	03.02.04	33.33	09.03.04	25.36	20.03.04	25.87
6	10.02.04	37.56	16.03.04	36.88	27.03.04	29.65
7	17.02.04	39.15	23.03.04	43.22	03.04.04	37.45
8	24.02.04	41.89	30.03.04	45.12	10.04.04	39.54
9	03.03.04	44.25	06.04.04	47.23	17.04.04	43.88
10	10.03.04	47.65	13.04.04	49.77	24.04.04	47.44
11	17.03.04	49.33	20.04.04	51.47	31.04.04	51.83

D/S – Date of sowing

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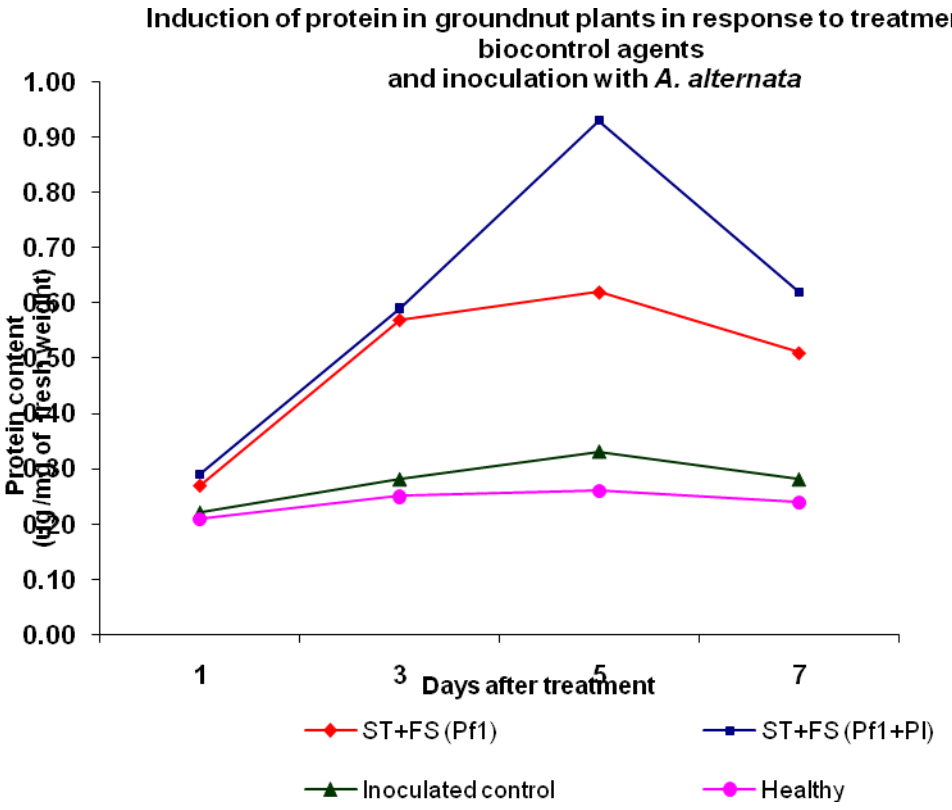
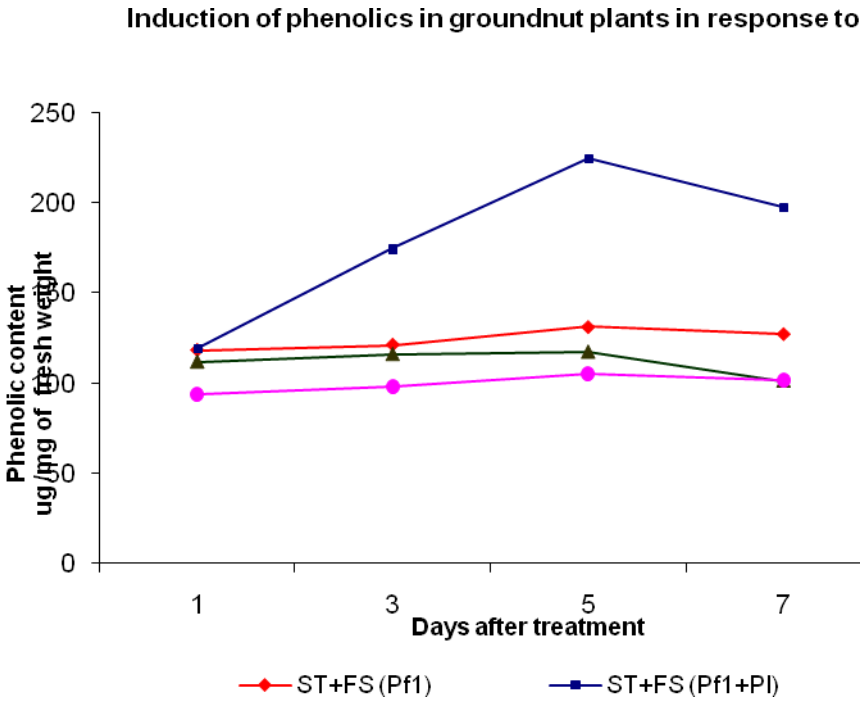
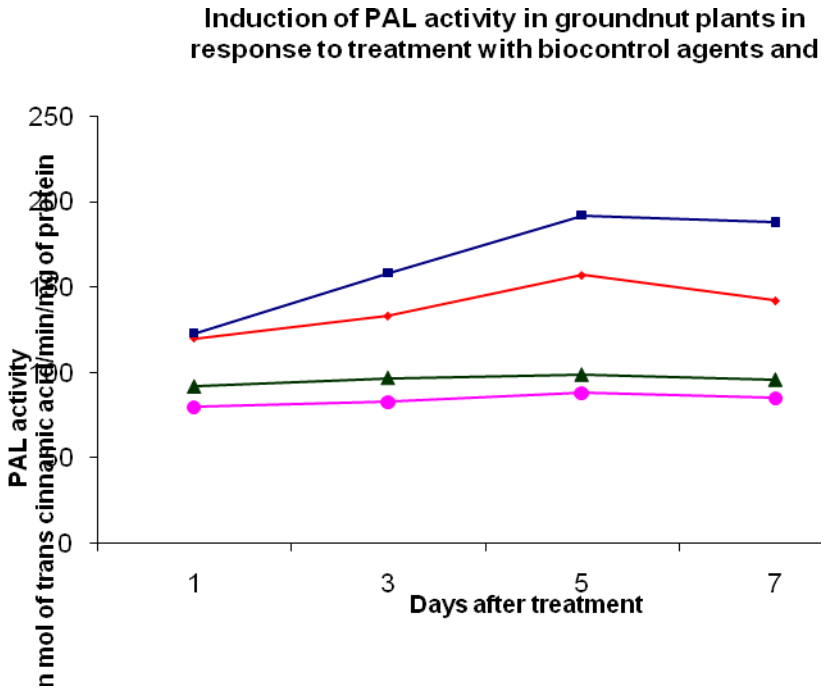
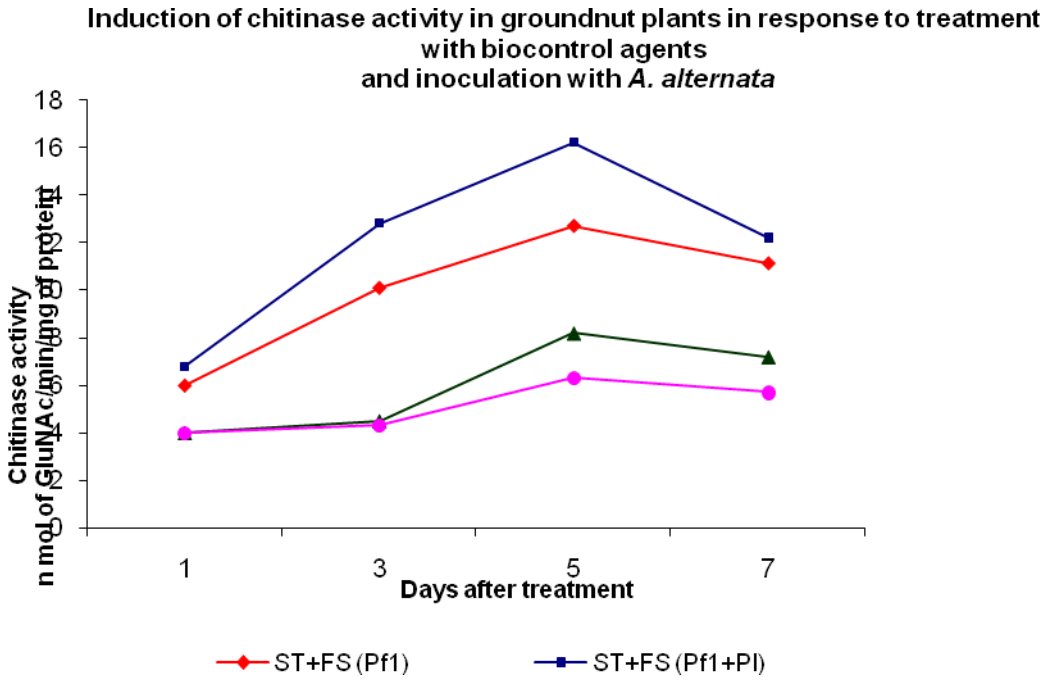
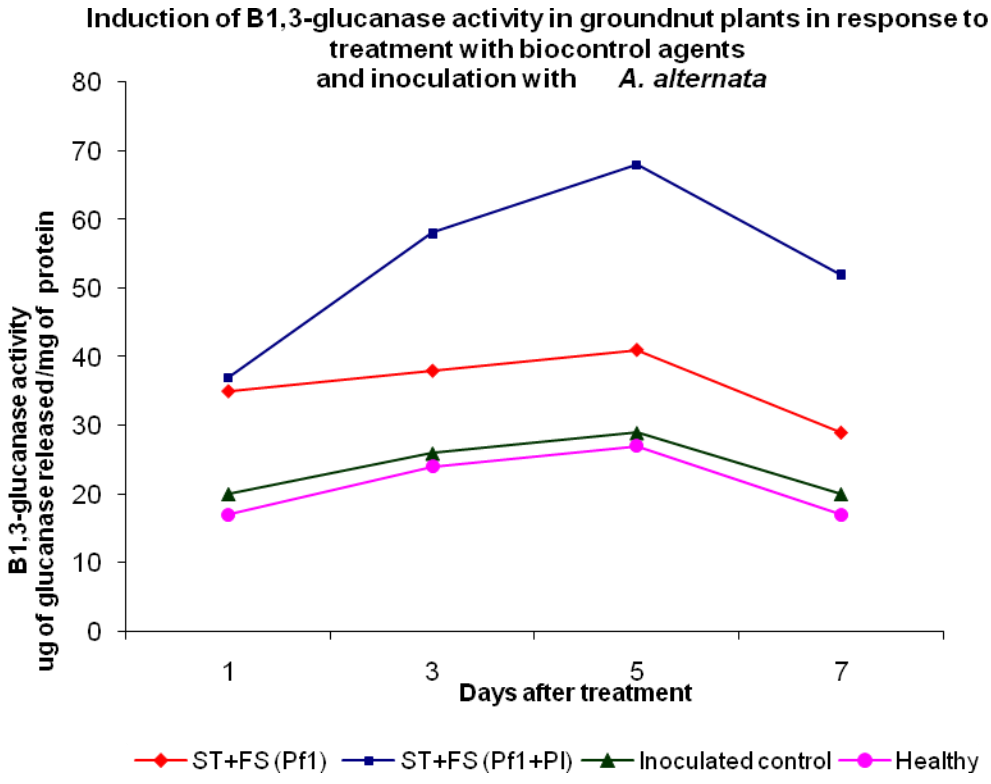
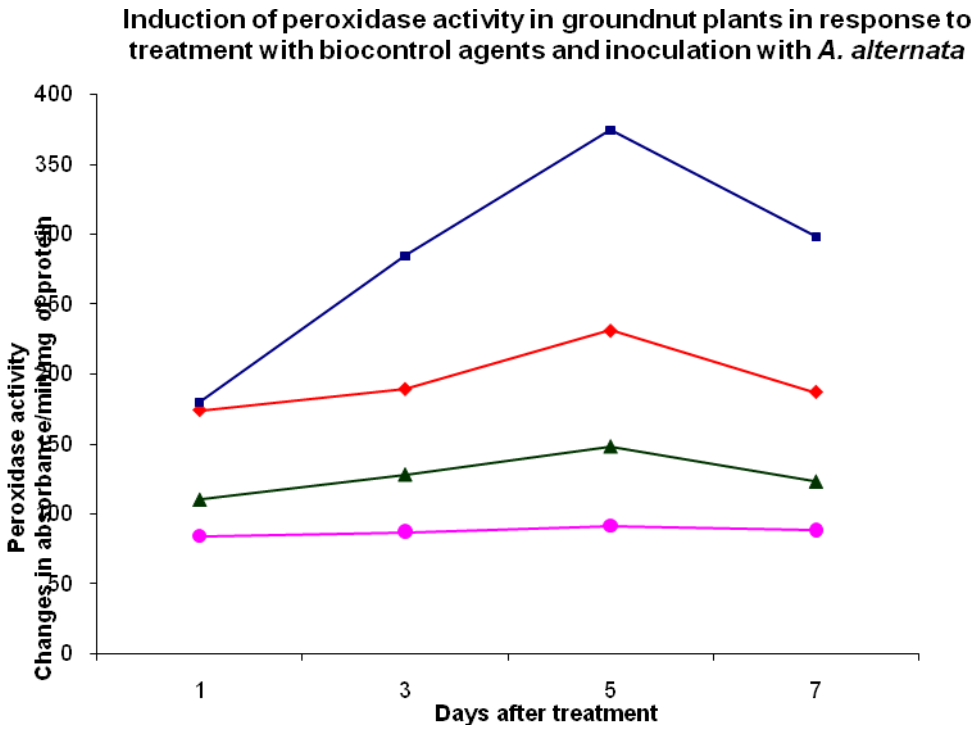

Induction of protein in groundnut plants in response to treatment with biocontrol agents
and inoculation with A. alternata
Protein content (µg /ml of fresh weight)
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
1
3
5
7
Days after treatment
ST+FS (Pf1)
ST+FS (Pf1+PI)
Inoculated control
Healthy

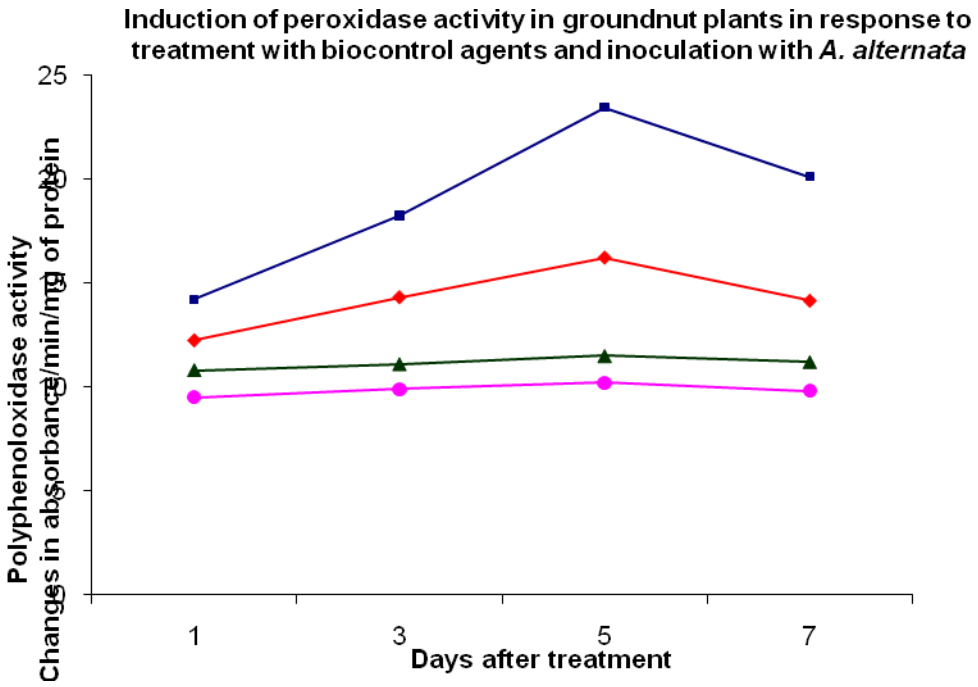

Induction of phenolics in groundnut plants in response to
250
200
150
100
50
0
Phenolic content
ug/mg of fresh weight
1
3
5
7
Days after treatment
ST+FS (Pf1)
ST+FS (Pf1+Pl)

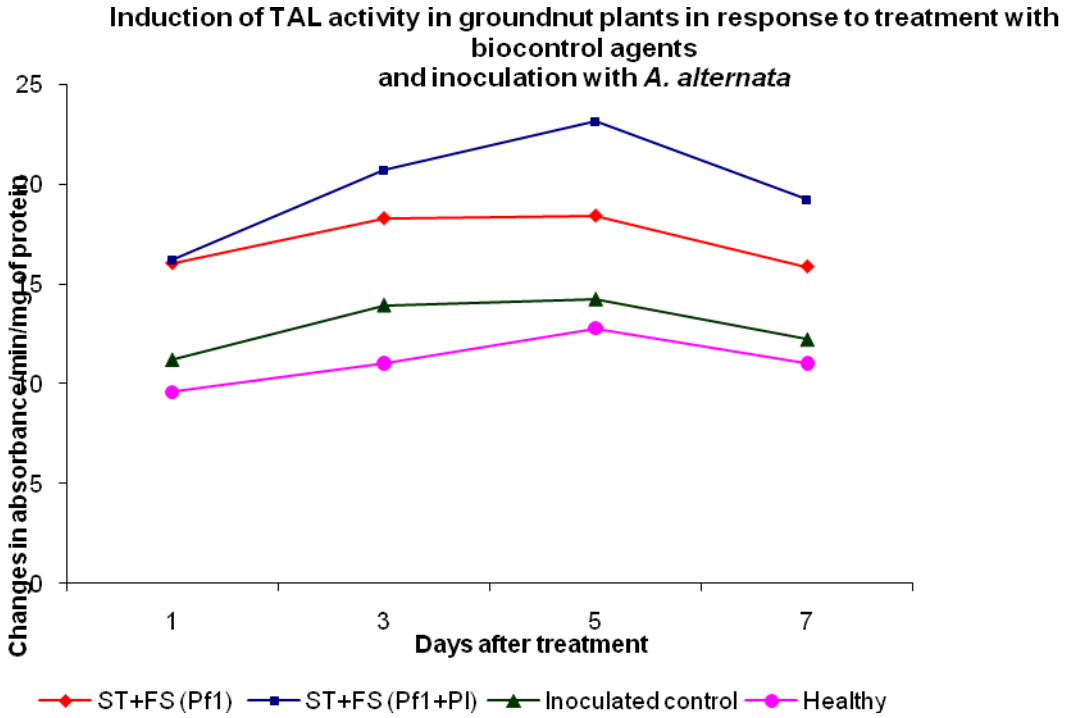

Induction of PAL activity in groundnut plants in response to treatment with biocontrol agents and
PAL activity
n mol of trans cinnamic acid/min/mg of protein
250
200
150
100
50
0
1
3
5
7
Days after treatment

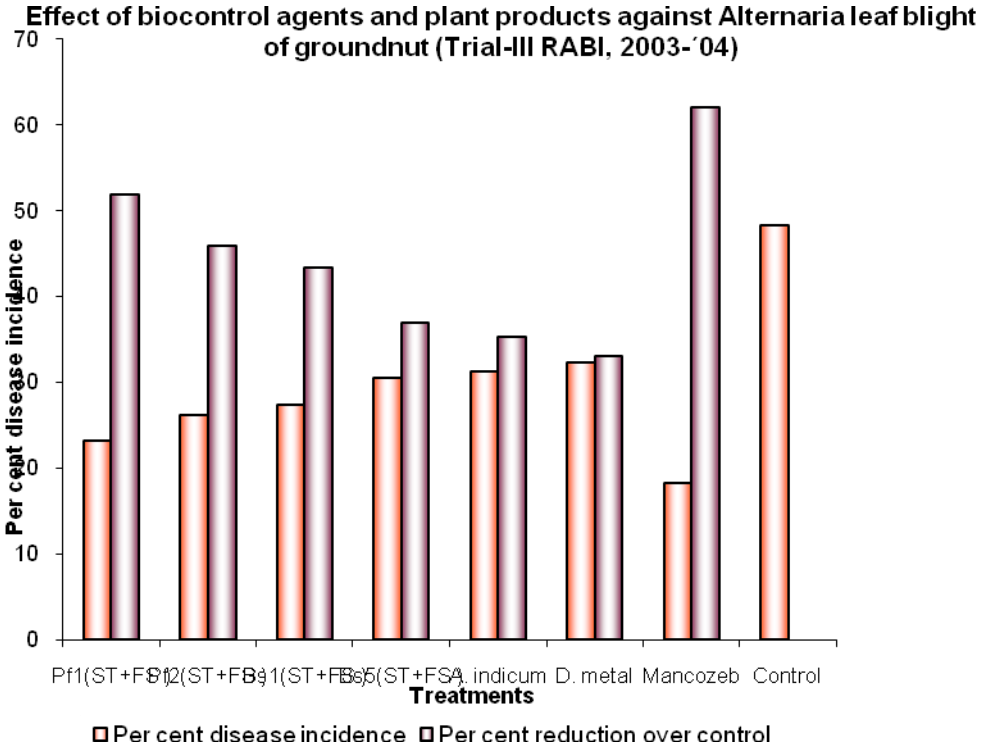

Induction of chitinase activity in groundnut plants in response to treatment with biocontrol agents and inoculation with A. alternata
Chitinase activity
n mol of GluNAc/min/mg of protein
18
16
14
12
10
8
6
4
2
0
1
3
5
7
Days after treatment
ST+FS (Pf1)
ST+FS (Pf1+Pl)

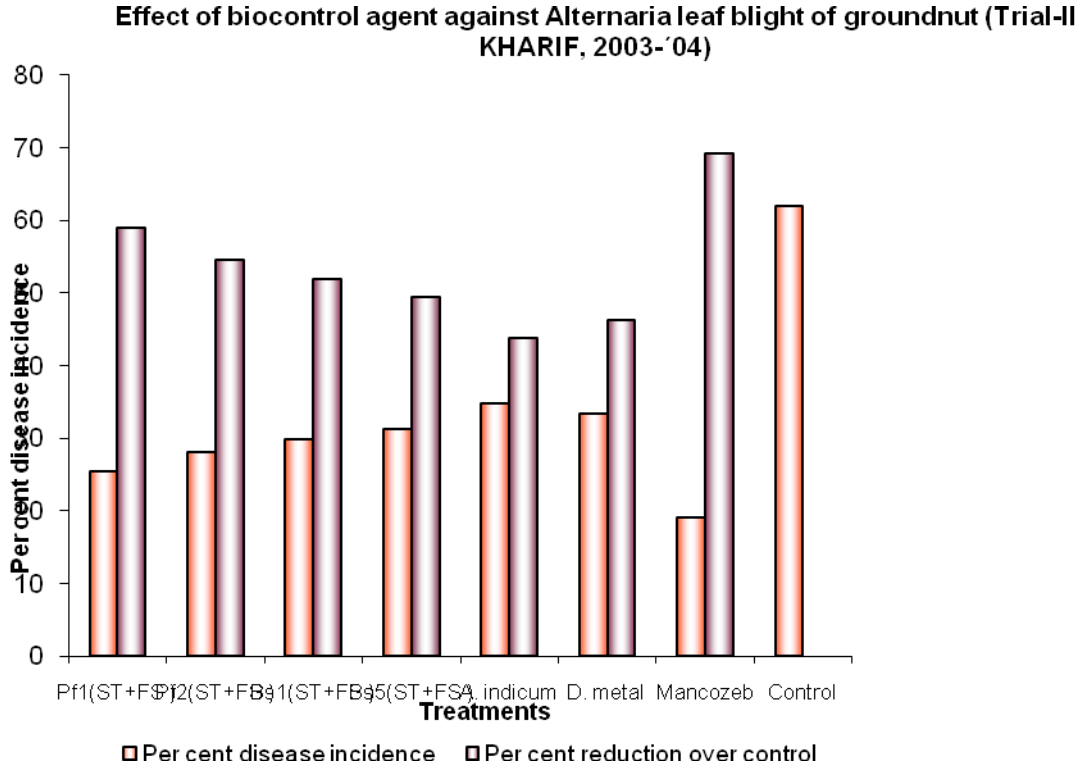

Induction of B1,3-glucanase activity in groundnut plants in response to treatment with biocontrol agents and inoculation with A. alternata
B1,3-glucanase activity ug of glucanase released/mg of protein
80
70
60
50
40
30
20
10
0
1
3
5
7
Days after treatment
ST+FS (Pf1)
ST+FS (Pf1+PI)
Inoculated control
Healthy


Induction of peroxidase activity in groundnut plants in response to treatment with biocontrol agents and inoculation with A. alternata
Peroxidase activity
Changes in absorbance/min/mg of protein
400
350
300
250
200
150
100
50
0
1
3
5
7
Days after treatment


Induction of peroxidase activity in groundnut plants in response to treatment with biocontrol agents and inoculation with A. alternata
Polyphenoloxidase activity
Changes in absorbance/min/mg of protein
25
20
15
10
5
0
1
3
5
7
Days after treatment


Induction of TAL activity in groundnut plants in response to treatment with biocontrol agents
and inoculation with A. alternata
Changes in absorbance/min/mg of protein
25
20
15
10
5
0
1
3
5
7
Days after treatment
ST+FS (Pf1)
ST+FS (Pf1+Pl)
Inoculated control
Healthy


Effect of biocontrol agents and plant products against Alternaria leaf blight of groundnut (Trial-III RABI, 2003-´04)
Per cent disease incidence
70
60
50
40
30
20
10
0
Pf1(ST+FS)
Pf2(ST+FS)
Bs1(ST+FS)
Bs5(ST+FS)
A. indicum
D. metal
Mancozeb
Control
Treatments
Per cent disease incidence
Per cent reduction over control


Effect of biocontrol agent against Alternaria leaf blight of groundnut (Trial-II KHARIF, 2003-´04)
Per cent disease incidence
80
70
60
50
40
30
20
10
0
Pf1(ST+FSA)
Pf2(ST+FSA)
Bs1(ST+FSA)
Bs5(ST+FSA)
A. indicum
D. metal
Mancozeb
Control
Treatments
Per cent disease incidence
Per cent reduction over control

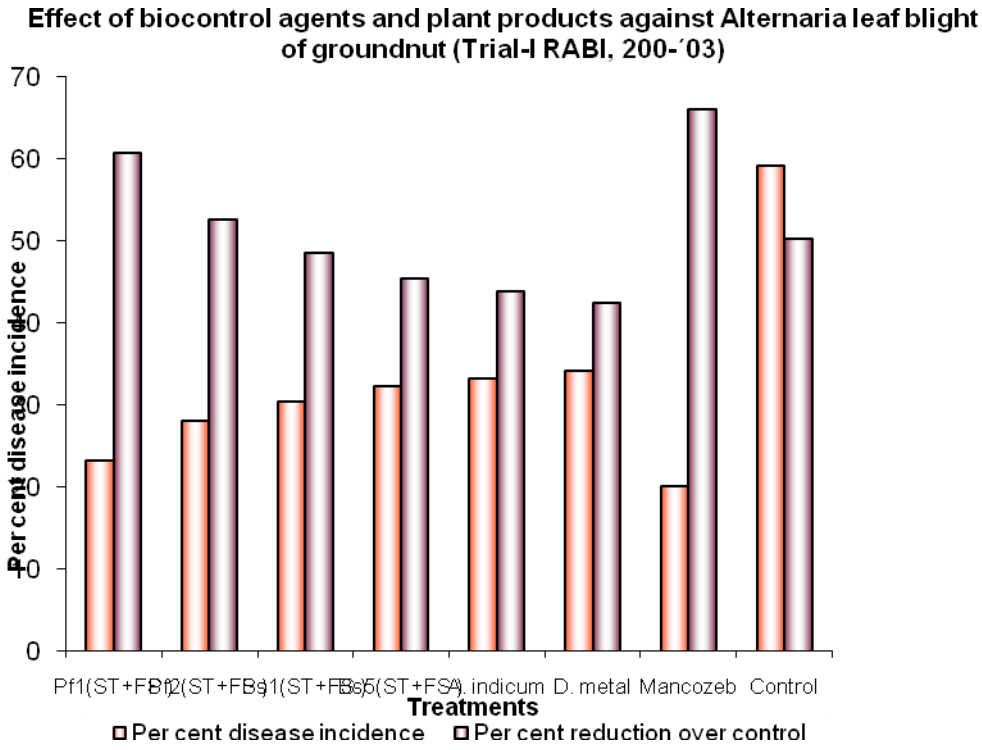

Effect of biocontrol agents and plant products against Alternaria leaf blight of groundnut (Trial-I RABI, 200-´03)
70
60
50
40
30
20
10
0
Per cent disease incidence
Pf1(ST+FSA)
Pf2(ST+FSA)
Bs1(ST+FSA)
Bs5(ST+FSA)
A. indicum
D. metal
Mancozeb
Control
Treatments
Per cent disease incidence
Per cent reduction over control

Fig.1 Induction of PAL activity in response to Pf1 treatment

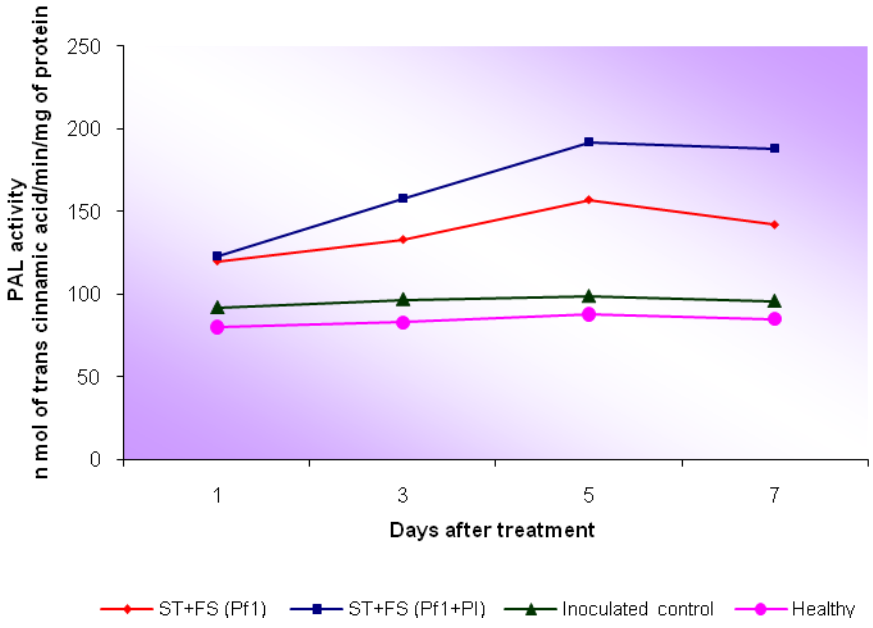


Fig.2 Induction of peroxidase activity in response to Pf1 treatment

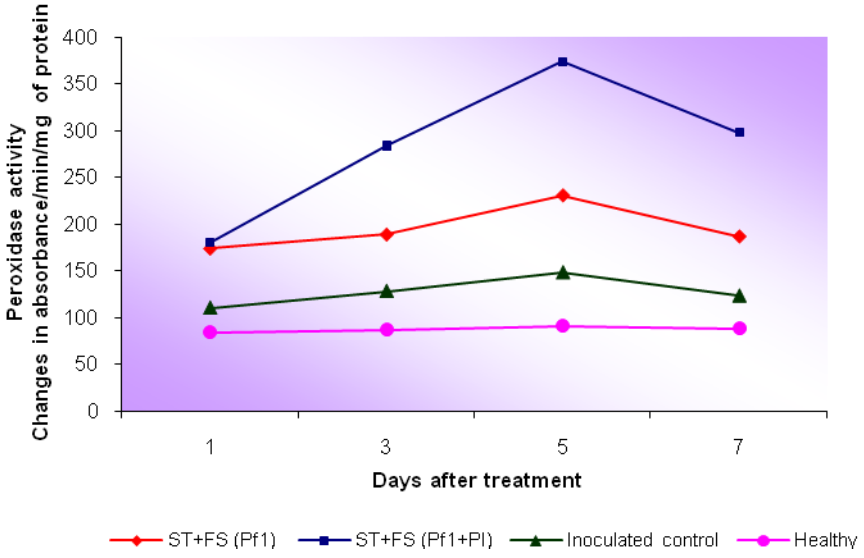


Fig.3 Induction of polyphenol oxidase activity in response to Pf1 treatment

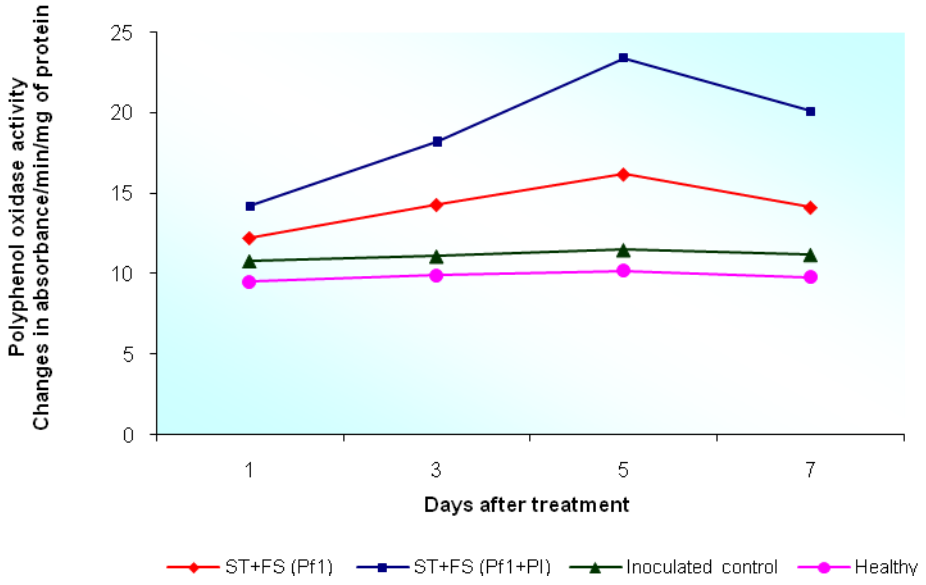


Fig.4 Induction of chitinase activity in response to Pf1 treatment

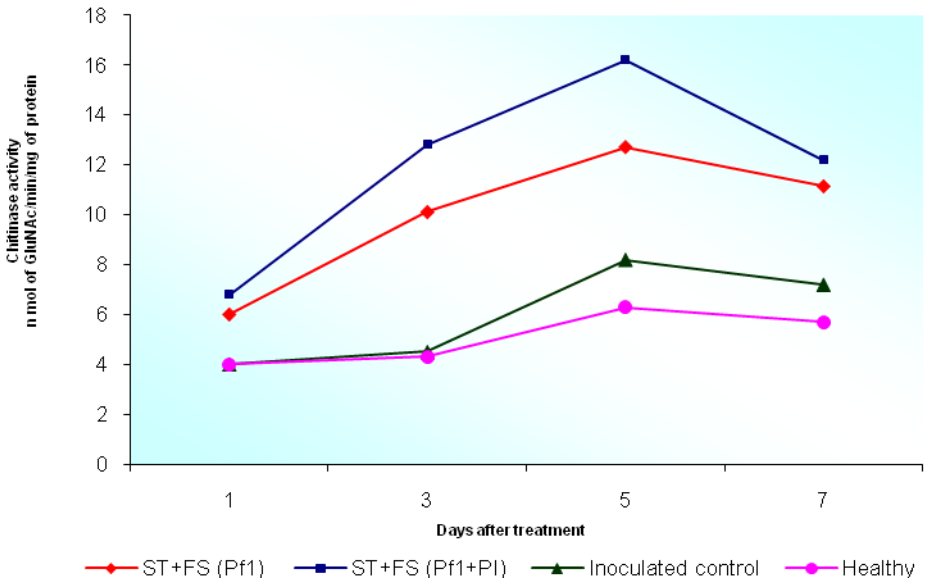
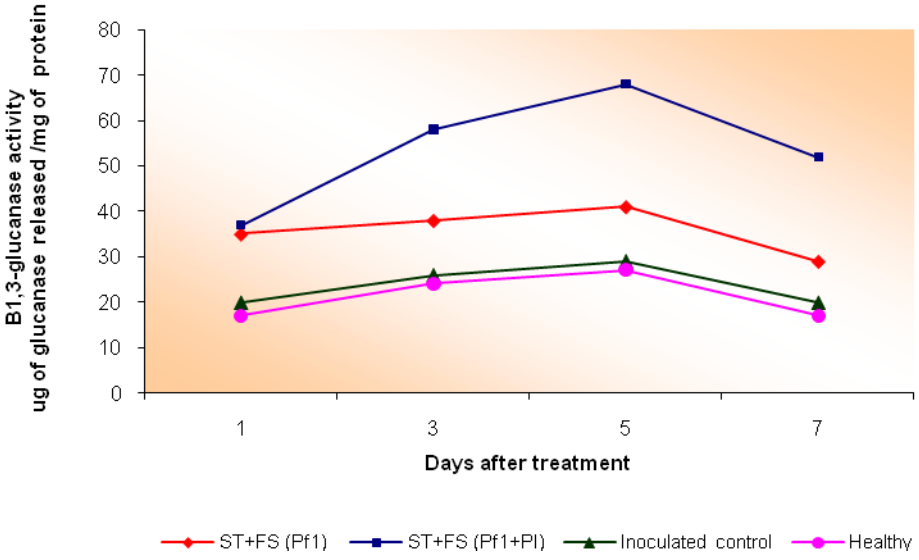


Fig. 5 Induction of B1,3-glucanase activity in response to Pf1 treatment



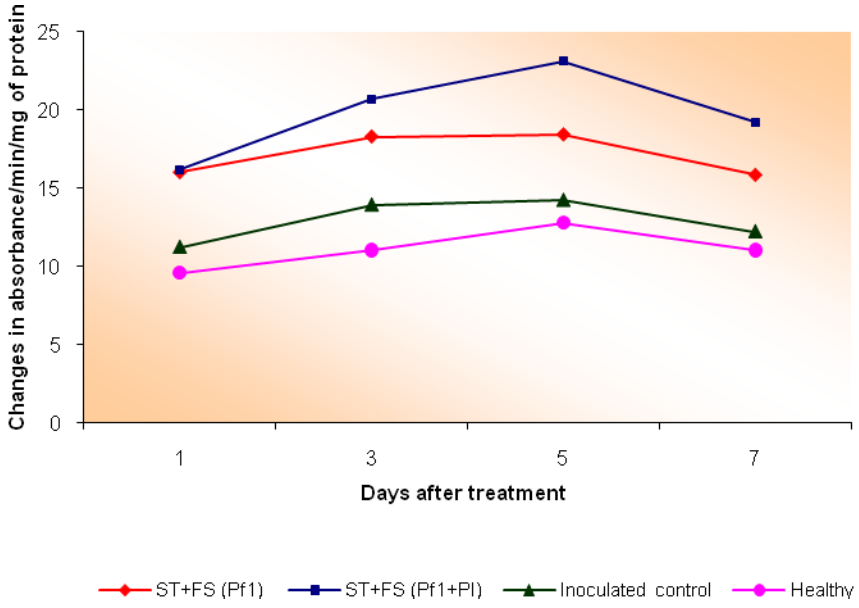

Changes in absorbance/min/mg of protein
25
20
15
10
5
0
1
3
5
7
Days after treatment
ST+FS (Pf1)
ST+FS (Pf1+PI)
Inoculated control
Healthy

Fig.7 Phenolics content in groundnut leaf treated with Pf1

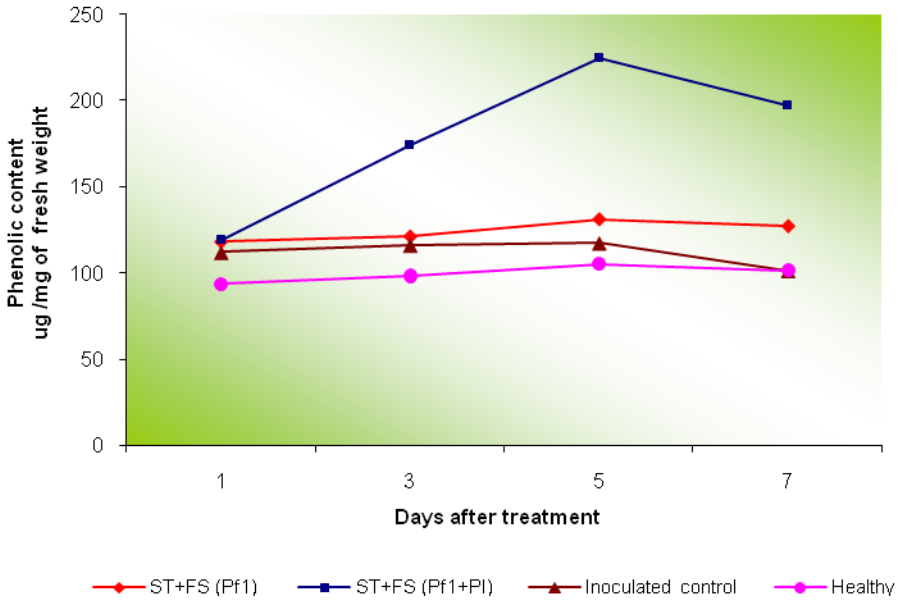


Fig 8. Protein content in groundnut leaf treated with Pf1

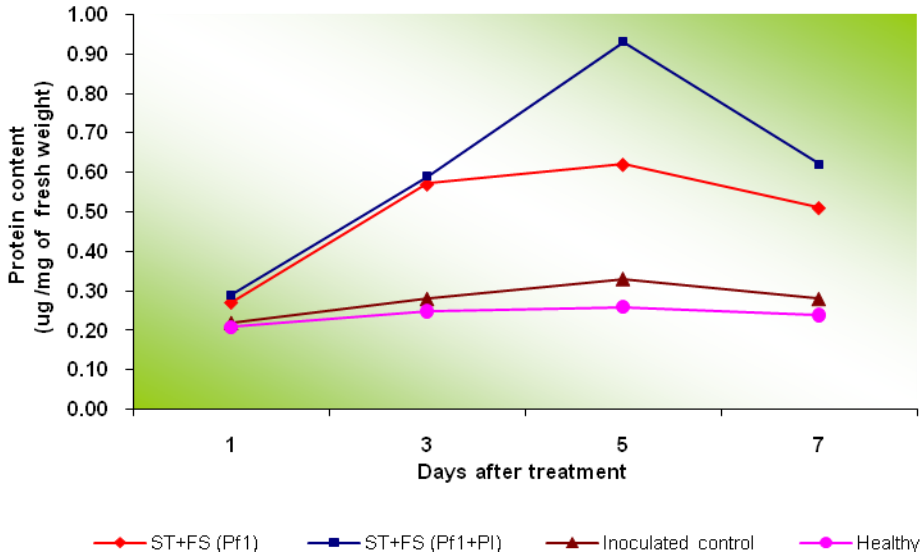


Fig.9 Effect of biocontrol agents and plant products against Alternaria leaf blight of groundnut (Trial-I Rabi, 200-´03)

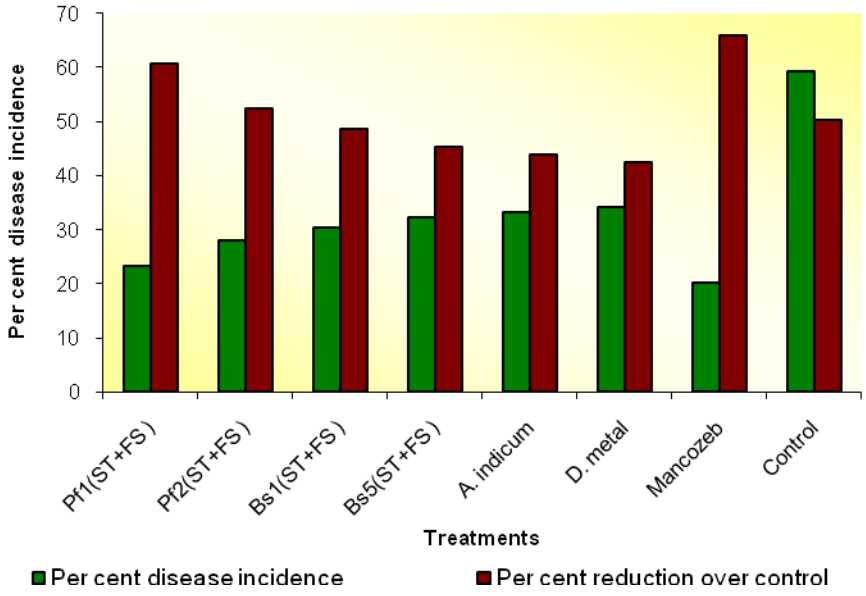


Fig. 10 Effect of biocontrol agent against Alternaria leaf blight of groundnut (Trial-II Kharif, 2003-´04)

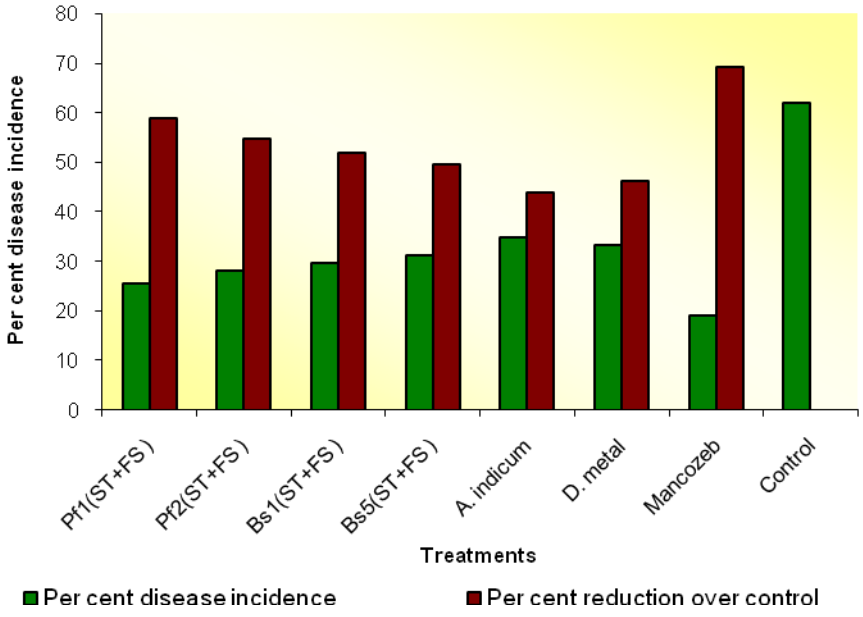
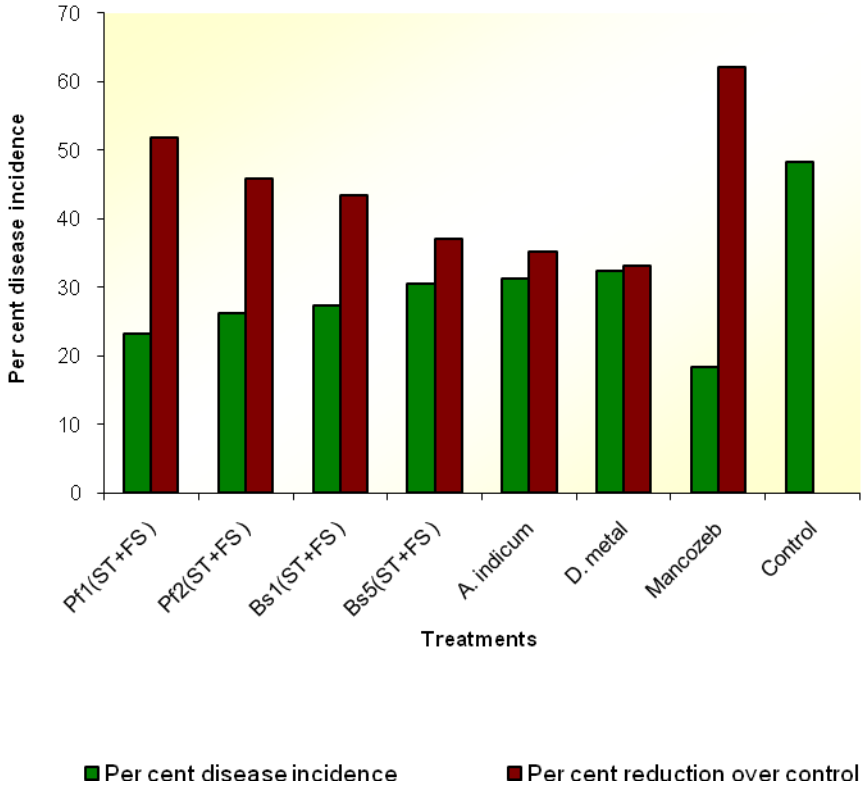
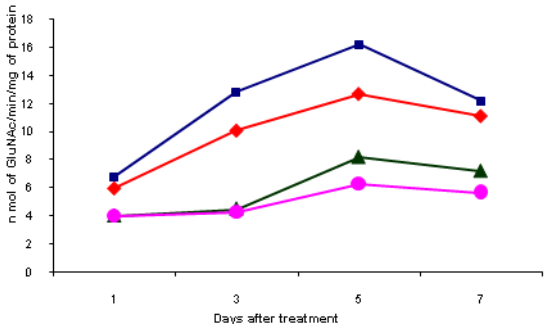


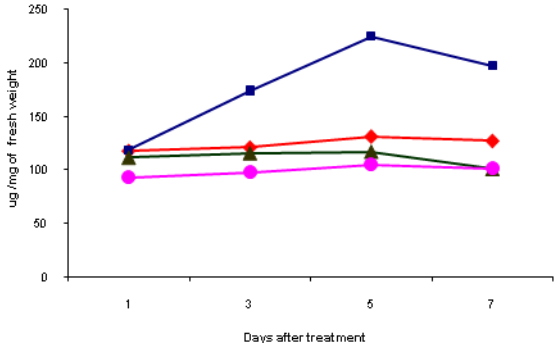
Fig.11 Effect of biocontrol agents and plant products against Alternaria leaf blight of groundnut (Trial-III Rabi, 2003-´04)



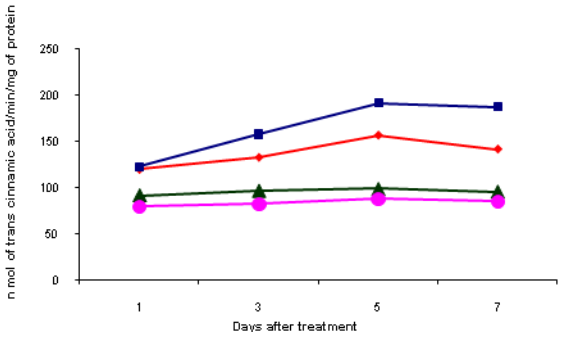
Induction of chitinase activity in groundnut plants in response to treatment with biocontrol agents, plant products and inoculation with *A. alternata*



Induction of phenolic content in groundnut plants in response to treatment with biocontrol agents, plant products and inoculation with A. alternata



Induction of phenylalanine ammonia lyase activity in groundnut plants in response to treatment with biocontrol agents, plant products and inoculation with A. alternata



Induction of protein content in groundnut plants in response to treatment with biocontrol agents, plant products and inoculation with *A. alternata*

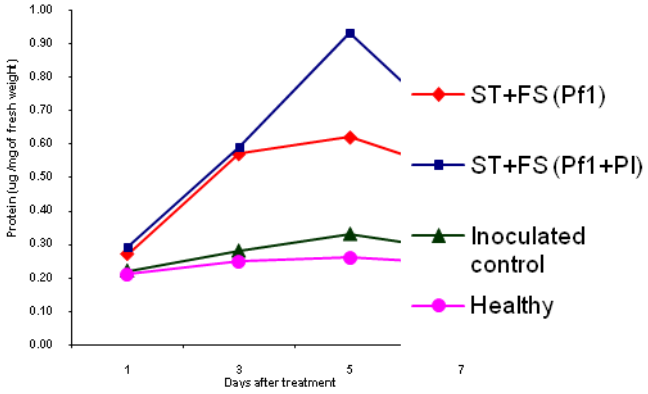


Plate 3. *In vitro* screening of *P. fluorescens* against *A. alternata*

Plate 4. *In vitro* screening of *B. subtilis* against *A. alternata*

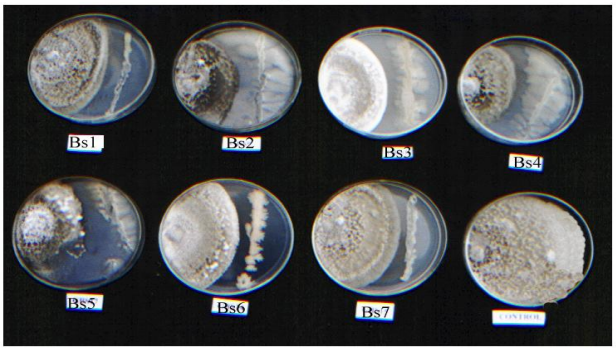
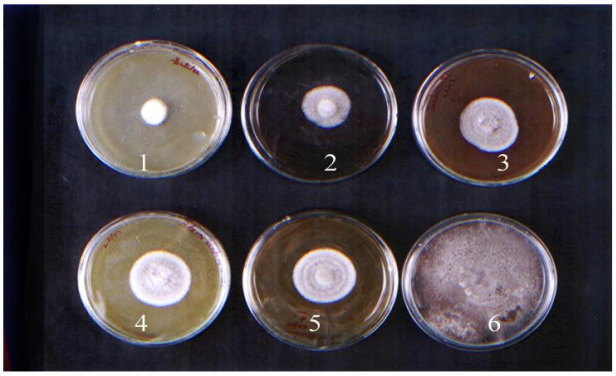


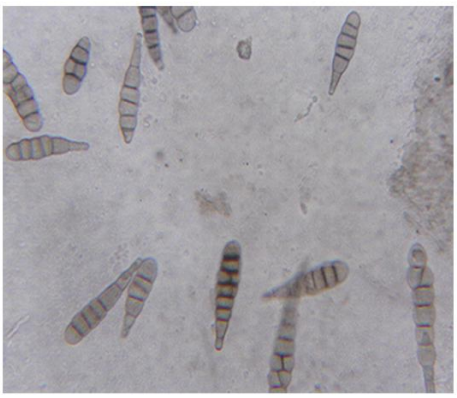
Plate 5. *In vitro* screening of leaf extracts against *A. alternata*



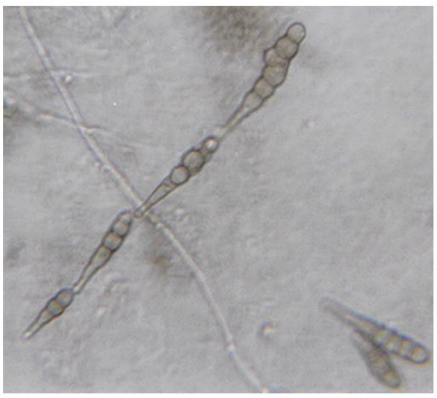
1-A. indicum
2-D. metel
3- L. inermis
4- A. indica
5- O. sanctum
6- Control

Plate 1. Symptom of *Alternaria alternata* leaf blight in groundnut

Plate 2. Microscopic view of *Alternaia alternata*



a. Individual conidia



b. Chains of conidia

Plant growth

Plate 6. Plant growth p[romoting activity by biocontrol agents

Plate 7. Production of HCN by *P. fluorescens*

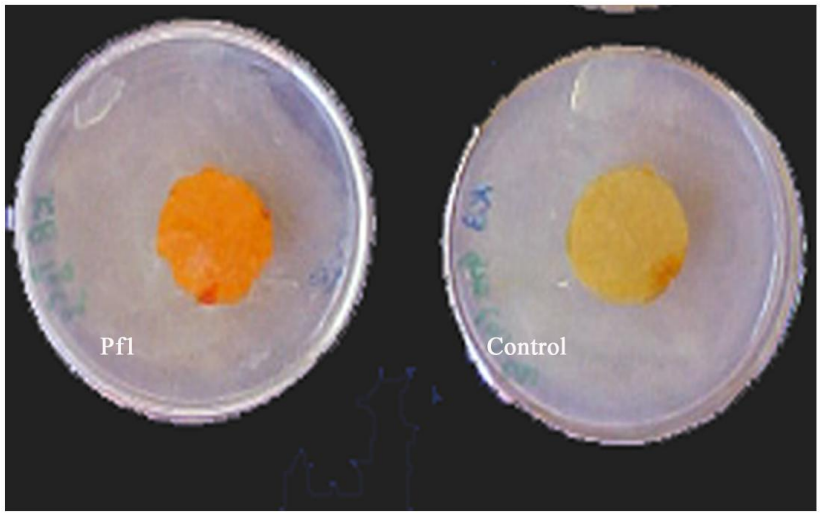


Plate 10. Induction of peroxidase isoforms in biocontrol agents treated plants

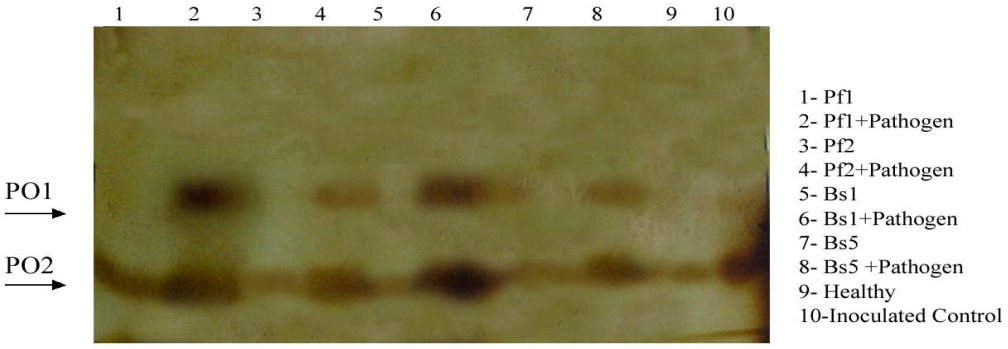


Plate 11. Induction of polyphenol oxidase isoforms in biocontrol agents treated plants

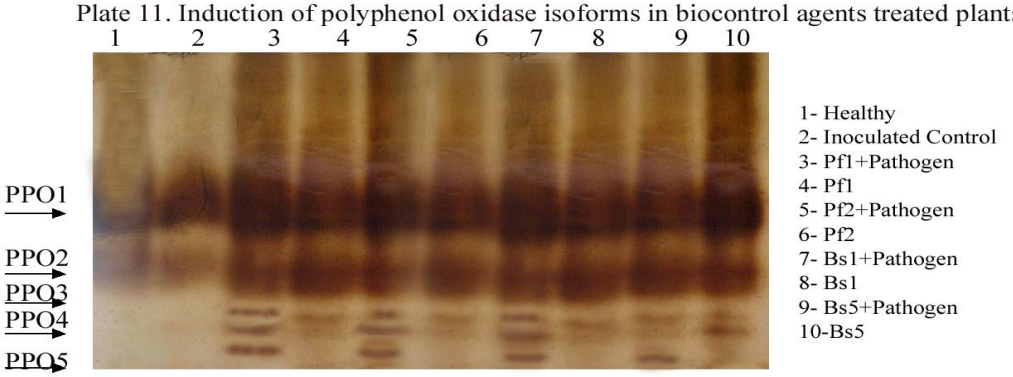


Plate 12. SDS PAGE analysis in biocontrol agents treated plants

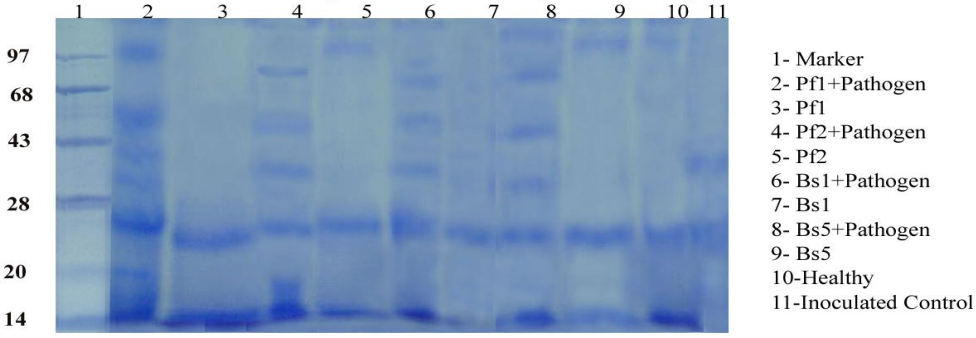


Plate 13. Field view of the experiment

Pf1 treated (ST+FS)

Control

Plate 14. Determination of optimum antiserum and antigen dilution by using indirect ELISA

Antiserum

Antigen

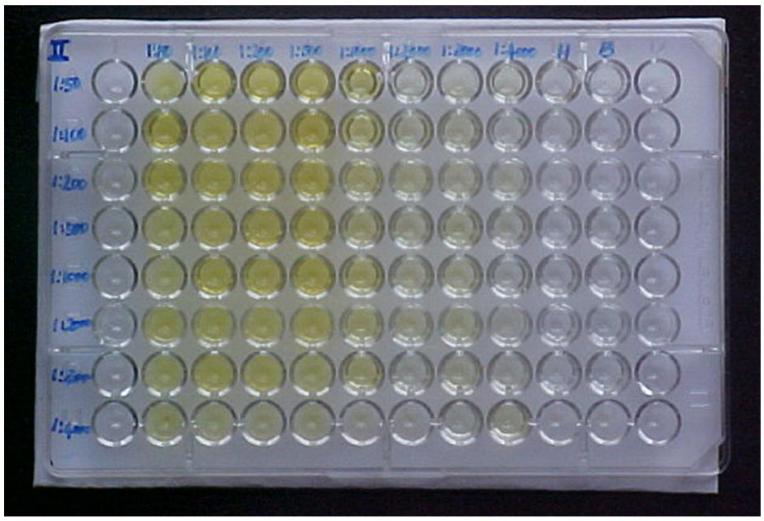
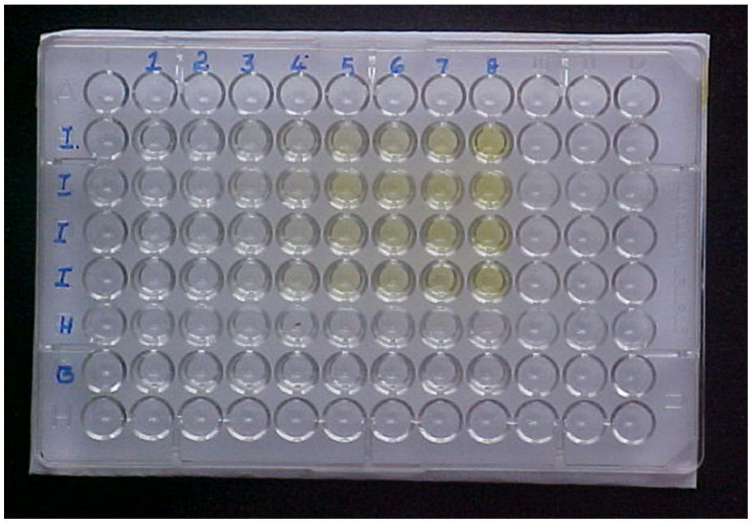


Plate 15. Detection of *A. alternata* in groundnut plants using indirect ELISA

Antiserum

Antigen

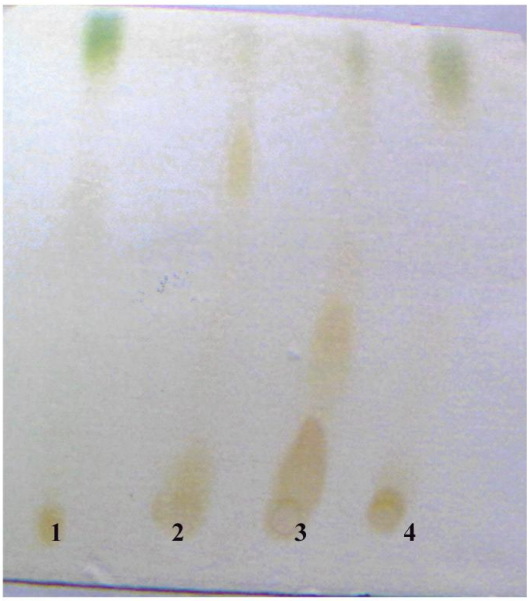


Antigen dilution - 1: 100
Antiserum dilution - 1:1000
I- Infected samples
H- Healthy samples

Plate 8. Separation of phenolic compounds from leaf extracts by TLC

1. *Abutilon indicum*
2. *Datura metel*
3. *Lawsonia inermis*
4. *Azadiracta indica*

Plate 9. Separation of alkaloid compounds from leaf extracts by TLC



1. *Abutilon indicum*
2. *Datura metel*
3. *Lawsonia inermis*
4. *Azadiracta indica*