RHIZOBACTERIA INDUCED SYSTEMIC RESISTANCE AGAINST BIOTROPH (*Exobasidium vexans*) AND NECROTROPH (*Macrophomina phaseolina*) PATHOGENS IN TEA AND GREEN GRAM

Thesis submitted in part fulfillment of the requirements for the degree of MASTER OF SCIENCE (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, "**RHIZOBACTERIA INDUCED SYSTEMIC RESISTANCE AGAINST BIOTROPH (*Exobasidium vexans* Massee) AND NECROTROPH (*Macrophomina phaseolina* (Tassi) Goid) PATHOGENS IN TEA AND GREEN GRAM**" submitted in part fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE (AGRICULTURE) IN PLANT PATHOLOGY** to the Tamil Nadu Agricultural University, Coimbatore, is a record of bonafide research work carried out by **Mr. D. SARAVANA KUMAR** 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.

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Abstract

ABSTRACT

RHIZOBACTERIA INDUCED SYSTEMIC RESISTANCE AGAINST BIOTROPH (*Exobasidium vexans*) AND NECROTROPH (*Macorphomina phaseolina*) PATHOGENS IN TEA AND GREEN GRAM

By

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A study was undertaken to test the efficacy of PGPR strains against the biotrophic and necrotrophic pathogens in tea and green gram both under greenhouse and field conditions. Among the PGPR strains tested under *in vitro* conditions against the necrotroph *Macrophomina phaseolina* in green gram, *Pseudomonas fluorescens* Pf1 was found to be effective in inhibiting the mycelial growth of the pathogen and also it was found to promote the vigour index of green gram seedlings. Among the various PGPR bioformulations tested, *P. fluorescens* Pf1 along with chitin bioformulation mixture was

found to be effective in reducing the disease incidence of root rot in green gram under both greenhouse and field conditions when compared to untreated control. Bioformulation mixture containing *P. fluorescens* and chitin significantly increased the pulse grain yield under both greenhouse and field conditions than the control and also this formulation significantly promoted the survival and colonization ability of Pf1 strain in the rhizosphere of green gram plants under field conditions. The same bioformulation mixture also enhanced the induction of the defense related enzymes *viz.*, phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, chitinase, β-1, 3-glucanase and phenols when plants were challenged with the pathogen.

A field trial conducted at Anamalais Hills for two seasons (2001 and 2002) to test the efficacy of PGPR strains against the blister blight disease in tea plantations indicated that foliar spray of *P. fluorescens* at weekly interval significantly reduced the disease incidence with corresponding enhancement in yield. The talc based formulation of *P. fluorescens* significantly increased the activity of defense related enzymes *viz.*, phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, chitinase, β-1, 3-glucanase and phenols when compared to untreated control. The efficacy of Pf1 formulation against blister blight pathogen was comparable with that of chemical treatment.

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CHAPTER I

INTRODUCTION

Tea (*Camellia sinensis* (L) O. Kuntze), the oldest known beverage is made from tender leaves of tea plant. India is the largest producer of tea, which accounts for 20 per cent of global area and constitutes 28 per cent of world production. The total area under tea plantation is 5.07 lakh ha. (Boriah, 2002). Production of tea is hindered by various biotic and abiotic stresses. Among the biotic factors, the fungal disease blister blight caused by *Exobasidium vexans* Massee causes severe damage accompanied by high yield losses (Subba Rao, 1946).

In a country like India where a large population constitutes vegetarians, the cheap and best source of protein are pulses. Pulses contribute an important ingredient in predominantly vegetarian Indian diet. In India, green gram is grown in an area of 3.05 m ha. with an annual production of 1.16 mt and contributes 20 per cent to overall pulse production in India. In Tamil Nadu, it is grown in an area of 1.22 lakh ha. with a production of 0.52 lakh tonnes (AICPIP, MULLaRP report, 2000). The production and productivity of grain yield is affected by major fungal and bacterial diseases. Among these, the fungal disease *viz.*, dry root rot caused by *Macrophomina phaseolina* (Tassi.) Goid., occupies the premier position.

Therapeutic approach of killing the above pathogens with toxic chemicals has been the prevailing disease control strategy for over fifty years. Though, fungicides shown promising results in controlling the biotroph (blister blight) and necrotroph (root rot) fungal pathogens, phytotoxicity and fungicide residues are the major problems besides causing environmental pollution and human health hazards. In this context,

biocontrol approaches help to develop ecofriendly control strategy for managing the pathogens in crop plants.

The utilization of plant's own defense mechanism is a fascinating arena of research which is being practiced all over the world to manage the plant diseases. Plants have latent defense mechanism against pathogens, which can be systemically activated upon exposure of plants to stress or infection by pathogens (Baker *et al.*, 1997). This phenomenon is called Induced Systemic Resistance (Tuzun and Kuc, 1991). The mechanism operates through the activation of multiple defense compounds at sites distant from the point of pathogen attack (Dean and Kuc, 1985). The inducers include pathogens (Dalisay and Kuc, 1995; Hammerschmidt, 1999), plant growth promoting rhizobacteria (PGPR) (Leeman *et al.*, 1995; Ramamoorthy *et al.*, 2002a), chemicals (Michael *et al.*, 2001) and botanicals (Singh *et al.*, 1990).

PGPR are the major root colonizers, belong to different genera and most reported strains are from species of *Pseudomonas, Bacillus* and *Serratia* etc. The strains of PGPR are known to survive both in rhizosphere and phyllosphere (Krishnamurthy and Gnanamanickam, 1998). Several *Pseudomonas* strains have been shown to induce ISR in plants against many fungal, bacterial and viral diseases (Chen *et al.*, 2000; Kloepper *et al.*, 1993; Krishnamurthy and Gnanamanickam, 1998; Vidhyasekaran and Muthamilan, 1999; Raupach *et al.*, 1996; Maurhofer *et al.*, 1998; Kandan, 2000).

Recent investigations on mechanisms of biological control by plant growth promoting fluorescent pseudomonads revealed that several strains protect the plants from pathogen attack by strengthening the epidermal and cortical cell walls with deposition of newly formed barriers beyond infection sites including callose, lignin and phenolics

(Benhamou *et al.*, 1996a, b, c; Duijff *et al.*, 1997; M'Piga *et al.*, 1997) and by activating defense genes encoding chitinase, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase (M'Piga *et al.*, 1997; Chen *et al.*, 2000) and enzymes which involved in the synthesis of phytoalexins (Maurhofer *et al.*, 1994; van Peer *et al.*, 1991; Ongena *et al.*, 1999) and expression of stress-related proteins (Timmusk and Wagner, 1999).

When *Pseudomonas* spp. mixed with some other strains or other bacterial antagonists, the biocontrol efficacy is increased (Duffy *et al.*, 1996; Pierson and Weller, 1994; Raupach and Kloepper, 1998). Mixing of chitin with the PGPR has also been found to increase the biocontrol efficacy (Nandakumar, 1998; Radja Commare, 2000; Viswanathan and Samiyappan, 2001a, b). In addition to disease control, fluorescent pseudomonads exert beneficial effect on plant growth promotion (Raupach and Kloepper, 1998; Dubeikovsky *et al.*, 1993; Kloepper *et al.*, 1980). In recent years, the use of PGPR as an inducer of systemic resistance in crop plants against different pathogens has been demonstrated under field conditions (Vidhyasekaran and Muthamilan, 1999; Viswanathan, 1999; Viswanathan and Samiyappan, 1999; Nandakumar, 1998; Wei *et al.*, 1991 and 1996).

When plants are treated with PGPR, their physiological and biochemical characters are changed. These changes make the plants less suitable to subsequent attack by pathogens and plants are benefited from induced responses by reducing subsequent pathogen load, though it is often difficult to show the benefits of PGPR experimentally. Induced resistance is frequently viewed as an alternative tactic to constitutive resistance. Hence, understanding of the relationship between constitutive and induced resistance will be important in defining how best to use induced resistance in agriculture. To use the

PGPR induced responses as an effective pathogen management tool, there is a need to evaluate the effect of induced responses on plant performance and yield under agricultural settings.

With this supportive background information, the following objectives have been formulated to evolve the efficaceous bioformulations against the biotroph and necrotroph pathogens in tea and green gram.

1. Isolation, screening and selection of effective PGPR strains *viz.*, *Pseudomonas fluorescens* and *Bacillus subtilis* under *in vitro* conditions.
2. Development of new talc-based bioformulations of PGPR strains *viz.*, *Pseudomonas fluorescens* and *Bacillus subtilis* in different combinations.
3. Testing the efficacy of bioformulations against blister blight of tea and dry root rot of green gram under greenhouse and field conditions.
4. Studying the mechanisms involved in disease resistance against blister blight and dry root rot pathogens in tea and green gram.
5. Studying the influence of PGPR strains on plant growth promotion under greenhouse (green gram) and field conditions (green gram and tea).

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

2.1. Introduction

Exobasidium vexans Massee is one of the most important devastating biotrophic pathogens which causes blister blight disease on tea leaves. Mann (1906) first reported the occurrence of blister blight disease from Assam. Further, the disease spread to the different parts of tea growing tracts all over the world. The outbreak of blister blight disease in South India was recorded during August, 1946 in the estates of Mundakayam, Peermade and in the Cumbum valley. Subba Rao (1946) reported the disease spread in Anamalais covering a distance of 100 km under the influence of South West monsoon winds.

Macrophomina phaseolina (Tassi) Goid is an important necrotrophic plant pathogen distributed worldwide and causes disease on more than 500 hosts (Sinclair, 1984). It's destructive nature on many crop plants has been well documented (Dhingra and Sinclair, 1978). It is one of the destructive plant pathogens known in the tropics and sub-tropics (Dhingra and Sinclair, 1978). It causes dry root rot in green gram plants under warm and dry weather conditions.

2.2. Importance of the diseases

2.2.1. Blister blight

The tea industry in South India suffered enormous crop losses in the early years of blister blight incidence from 1948 to 1962 (Subba Rao, 1934). The annual losses amounted to 18 m kg of tea before the control measures were fully used on extensive scale (VenkataRam, 1968). Blister blight inflicted severe crop losses both in Sri Lanka (Loos, 1950) and Indonesia (Schweizer, 1950). In a study in Sri Lanka, a crop loss of 33 per cent

was recorded in unprotected areas as compared to the fields which are sprayed with chemicals (de Silva *et al.*, 1977). It has been established that failure to control blister blight would lead to an annual yield loss of 43 per cent (Ordish, 1952). Venkata Ram (1964) reported 50 per cent of yield loss annually and tea made from blistered shoots appeared flaky. The pathogen attacks harvestable tender shoots, inflicting an enormous yield loss of 40 per cent and quality deterioration is noticed even below the 35 per cent disease threshold level (Gulati *et al.*, 1993).

2.2.2. Dry root rot

Root rot caused by *M. phaseolina* is a polyphagus soil-borne pathogen causing severe damage to green gram raised in different agro-climatic zones. The pathogen is known to survive in soil for prolonged periods in the form of dormant sclerotia as well as actively growing mycelium (Short *et al.*, 1989). Meyer *et al.* (1973) reported that *M. phaseolina* is a warm and dry weather pathogen resulting in 10 to 80 per cent crop loss.

2.3. Symptomatology

2.3.1. Blister blight

The pathogen usually infects the leaves and causes tiny translucent spots within three to ten days of infection, which later enlarges and become depressed on the upper surface of the leaves. Simultaneously, the underside of the leaf becomes convex to form the typical blister lesion. These blisters turn necrotic and lead to dieback. Sporulation is initiated only after formation of spores and spores are released into the atmosphere due to the rupture of epidermis (Rajalakshmi and Ramarethinam, 2000). The blister in the early stages is slightly brown but as the fungus begins to sporulate, it assumes a grey color and later as spores mature, it has a doughy white appearance. After the cessation of spore production, the blister becomes brown, dark and finally dries up evenly (Subba Rao, 1946). In addition to all these morphological changes several physiological alterations also takes place in response to

infection. It produces some enzymes that degrades the polysaccharides of the cell wall and thereby gain entry into the cell (Albersheim *et al.*, 1969).

2.3.2. Dry root rot

Saksena *et al.* (1970) observed leaf infection and seedling blight caused by *Rhizoctonia bataticola* (Taub) Butler. *M. phaseolina* caused complex disease syndrome like root rot, charcoal rot, seedling blight, foliage blight, tuber decay, dry rot, fruit rot, pod rot and seed rot in several crops in different parts of the world. The disease caused by *M. phaseolina* was more prevalent under dry and water stress conditions (Dhingra and Sinclair, 1975). Chaudhuri and Ahmed (1977) stated that under drought conditions the pathogen caused premature drying and blightening of infected seedlings. They also reported that in mature plants the typical symptoms were root and basal stem rot with a large number of minute sclerotia of the fungus under the bark and drying up of infected plants. Kannaiyan *et al.* (1980) reported the seed and soil-borne nature of the pathogen. Kannaiyan *et al.* (1984) stated that the disease incidence was severe in off- season and irrigated summer crops in different parts of India.

2.4. Pathogen characters

2.4.1. *Exobasidium vexans*

The pathogen was identified by Massee (1898) of the Kew Botanic Gardens and given the name, *Exobasidium vexans* Massee. The fungus belongs to Exobasidiales, Hymenomycetes and Basidiomycotina (Hawksworth *et al.*, 1983). The pathogen is a biotroph and there is no alternate host and the life cycle is completed on tea itself. The blister comprises numerous compact bundles of hyphae that forms the hymenial layer. The clavate basidia are produced on maturation. Generally two, occasionally three and rarely even four sterigmata are formed on basidium and bear a basidiospore. The spores are hyaline, single celled when immature and uniseptate at maturity. The basidiospores are disseminated by

wind and air dispersed basidiospores are the sole infective propagules in the life cycle of the fungus, inducing the development of hypertrophied and white powdery blisters in the susceptible young leaves.

2.4.2. *Macrophomina phaseolina*

The pathogen is necrotroph in nature and belongs to Sphaeropsidales, Coelomycetes and Deuteromycotina. It causes severe disease under dry and warm conditions, with soil being the primary source of inoculum (Dhingra and Sinclair, 1978). Sclerotia are the most important propagules for the survival of *M. phaseolina* in soil. Sclerotia are produced within roots, stems, leaves, fruits and easily on potato dextrose agar medium, which are black, smooth, hard, formed of dark brown, thick-walled cells. They are also formed in the host during parasitic phase and released into the soil as the host tissues decay (Cook *et al.*, 1973; Meyer *et al.*, 1974).

2.5. Management of biotrophs with biocontrol agents

Umesha *et al.* (1998) reported the effective control of downy mildew pathogen of pearl millet using *Pseudomonas fluorescens* either as seed treatment or as foliar application. According to Liu *et al.* (1995a, b) controlling mechanism might be due to the production of antibiotics or related substances by *P. fluorescens.*

Under field conditions, *Ampelomyces quisqualis* was used as a potential bioagent to control the powdery mildew of cucumber, carrot and mango and it significantly reduced the disease incidence (Sztejnberg *et al.*, 1989). Foliar application of *A. quisqualis* also controlled the powdery mildew of grapes (Falk *et al.*, 1995), *Dalbergia sissoo* (Rajasab and Vidyasagar, 1992), *Medicago lupulina* and *Lupinus polyphyllus* (Hijwegen and Buchenaur, 1984).

Singh *et al.* (2000) revealed that the seed treatment alone or in combination with aerial spray of plant growth promoting rhizobacterial (PGPR) cell suspensions strongly reduced the disease incidence of powdery mildew in pea plants under field conditions.

Dehne *et al.* (1984) observed that the wheat plants treated with *Bacillus subtilis* or its metabolic products induced a high level of resistance against *Erysiphe graminis* f. sp. *tritici*. *Bacillus thuringiensis* treated coffee leaves infected with *Hemileia vastatrix* enhanced the induced systemic resistance (ISR) which in turn lowered the disease incidence (Dagmar *et al.*, 1989).

Conspicuously, Enebak and Carey (2000) reported that PGPR can be used effectively to reduce the infection of an obligate rust pathogen on pine host and indicted that PGPR mediated systemic protection occurs for fusiform rust. Application of bacterial strains *viz.*, *Pantoea agglomerans* B1, *Stenotrophomonas maltophila* C3 has also given the effective control of bean rust both under greenhouse and field conditions (Yuen *et al.*, 2001).

There are also numerous reports as reviewed by Gowdu and Balasubramanian (1988) of bacterial strains in the genera, *Bacillus* and *Pseudomonas*, which are antagonists of rust fungi.

2.6. Management of necrotrophs with biocontrol agents

Use of antagonistic organisms against *Macrophomina* root-rot has been well documented (Lockwood, 1985; Mukhopadhyay *et al.*, 1989; Raguchander *et al.*, 1995) in several crops. Heterotrophic rhizobacteria of *P. fluorescens* types have been successfully used for biological control of several plant pathogens (Howell and Stipanovic, 1979; Weller and Cook, 1983; Ganesan and Gnanamanickam, 1987). A combination of seed and soil application of fluorescent pseudomonads for soil borne disease management was reported in chickpea (Vidhyasekaran and Muthamilan, 1995), pigeonpea (Vidhyasekaran *et al.*, 1997b).

Fluorescent pseudomonads isolated from suppressive soil were able to induce suppressiveness in conducive soil for crown and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Lemanceau and Alabouvette, 1991). In cotton, seedling disease caused by *Rhizoctonia solani* and *Pythium ultimum* was suppressed by application of

Pseudomonas spp. (Hagedorn *et al.*, 1993). Similarly, application of endophytic bacteria by stem injection reduced the root rot caused by *R. solani* and vascular wilt caused by *F. oxysporum* f. sp. *vasinfectum* (Chen *et al.*, 1995). Seed treatment with *P. fluorescens* strain 63-28 restricted the growth of *P. ultimum* (Benhamou *et al.*, 1996a, b) and *F. oxysporum* f. sp. *lycopersici* in tomato (M'Piga *et al.*, 1997). Seed treatment and soil application of *P. fluorescens* reduced root rot of blackgram caused by *M. phaseolina* (Shanmugam *et al.*, 2001) and panama wilt of banana caused by *F. oxysporum* f. sp. *cubense* (Raguchander *et al.*, 1997).

Jayashree *et al.* (2000) reported that application of *P. fluorescens* strain Pf1 as seed treatment followed by soil application effectively reduced the black gram root rot incidence. *P. fluorescens* isolate Pf1 was effective in reducing the damping-off incidence in tomato and hot pepper in greenhouse and field conditions and also it was found to protect tomato plants from wilt disease caused by *Fusarium oxysporum* f. sp. *lycopersici* (Ramamoorthy *et al.*, 2002b).

Gupta *et al.* (2002) stated that plant growth promoting bacterial strain, fluorescent pseudomonads showed a strong antagonistic effect against *M. phaseolina*, the charcoal rot pathogen of peanut.

2.7. PGPR as potent biocontrol agent

PGPR are a group of free-living microorganisms, which are the subset of rhizosphere bacteria known to aggressively colonize roots (Schroth and Hancock, 1982). They survive in seed or soil, multiply in the spermosphere in response to seed exudates rich in carbohydrates and amino acids (Kloepper *et al.*, 1992) attach to the root surface (Suslow, 1980) and become endophytic by colonizing in root cortex region. Rhizobacteria are distributed in the rhizosphere in a lognormal pattern and are sporadically dispersed along roots (Bahme and Schroth, 1987). Normally most of the reported PGPR strains are from *Pseudomonas* and *Bacillus* spp.

Biological control by antagonistic organisms has been tried extensively and some bacterial strains are emerged as potential biocontrol agents for the control of root and foliar diseases (Anuratha and Gnanamanickam, 1990; Raupach and Kloepper, 1998; Ramamoorthy *et al.*, 2002b). PGPR are also having the ability to protect above ground plant parts against viral, fungal and bacterial diseases by induced systemic resistance (ISR) (Kloepper *et al.*, 1992).

Among the PGPR, fluorescent pseudomonads are the most exploited bacteria for biological control of soil borne and foliar plant pathogens. In addition to disease control, they also promote the growth and development of crop plants. They can survive in both rhizosphere (Parke *et al.*, 1991) and phyllosphere (Wilson *et al.*, 1992). In the past three decades numerous strains of fluorescent pseudomonads have been isolated from the soil and plant roots by several workers and their biocontrol activity against soil-borne and foliar pathogens was reported (Austin *et al.*, 1977; Mew and Rosales, 1986; Rosales *et al.*, 1993; Rabindran and Vidhyasekaran, 1996, Vidhyasekaran and Muthamilan, 1999; Nandakumar *et al.*, 2001; Viswanathan and Samiyappan, 2001a, b; Ramamoorthy *et al.*, 2002a, b).

Many green house and field studies have been conducted to show the efficacy of fluorescent pseudomonads strains in the management of plant diseases. Cook and Rovira (1976) reported that the take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* was suppressed by certain strains of fluorescent pseudomonads which were isolated from the natural soil.

The practice of furrow application of *P. fluorescens* in cotton at the rate of 14.1 ml/m, improved the seedling stand (Hagedorn *et al.*, 1993). Chen *et al.* (1995) reported that application of endophytic bacteria brought about significant reduction of *F. oxysporum* f. sp. *vasinfectum* in cotton. Red rot in sugarcane caused by *Colletotrichum falcatum* was suppressed by certain strains of fluorescent pseudomonads (Viswanathan and Samiyappan, 1999).

Similarly, Jeyalakshmi *et al.* (1998) reported the possibility of managing the fruit rot and die back diseases of chilli by using *B. subtilis* as a biocontrol agent. Similar observation was reported by Rajavel (2000) in chilli against dieback disease.

The phyllosphere microbial community is an open system. Biocontrol specifically in the phyllosphere has been extensively reviewed since 1980 (Andrews, 1990). Sukumar and Ramalingam (1986) reported that leaf spot disease caused by *Cercospora moricolaw* in mulberry was reduced due to the application of *P. maltophila.*

Soilborne bacteria colonize pear flowers and gives control of fireblight almost as good as commercial bactericides (Thomson *et al.*, 1976). Spurr and Knudsen (1985) used bacteria from soil and other habitats for the control of *Alternaria* and *Cercospora* leaf spots of tobacco.

Foliar application of PGPR strains against the bacterial spot (*Xanthomonas axonopodis* pv. *vesicatoria*) in tomato, angular leaf spot (*Pseudomonas syringae* pv. *lachrymans*) in cucumber, blue mold (*Peronospora tabacina*) and wild fire (*P. syringae* pv. *tabaci*) in tobacco were found to be effective in controlling the disease incidence rather than drench application (Reddy *et al.*, 1999).

Saha *et al.* (2001) reported that cotton phylloplane bacteria belonging to genus *Pseudomonas* having the antagonistic activity against the most predominant and virulent race of *Xanthomonas campestris* pv. *malvacearum* under *in vitro* conditions and it was capable of reducing pathogen population drastically by colonizing the active multiplication sites of cotton leaves.

In addition to green house conditions, the PGPR mediated ISR against soil borne pathogens has been demonstrated under field conditions also (Nandakumar *et al.*, 2001; Ramamoorthy and Samiyappan, 2001; Vidhyasekaran and Muthamilan, 1995). Bacterial wilt caused by *Erwinia tracheiphila* was significantly reduced under field conditions (Kloepper *et al.*, 1993). Seed treatment of radish with resistance inducing *P. fluorescens* strain WCS374 reduced *Fusarium* wilt in naturally infested field soil upto 50 per cent (Leeman *et al.*, 1995).

2.8. Mechanism of action of PGPR on plant pathogenic fungi

2.8.1. Plant growth promotion

The studies on mechanism of growth promotion indicated that PGPR promote plant growth directly by production of plant growth regulators or indirectly by stimulating nutrient uptake, by producing siderophores or antibiotics to protect plants from soil borne pathogens or deleterious rhizosphere organisms. These *Pseudomonas* spp. may increase plant growth by producing gibberellin-like substances (Brown, 1972), mineralizing phosphates (Kavimandan and Gaur, 1971) or by other mechanisms which are not clearly understood. Barea *et al.* (1976) observed 50 phosphate solubilizing bacteria positive for IAA, gibberellin and cytokinin production. Among them, 17 isolates of *Pseudomonas* produced auxin or cytokinin and gibberellin.

Seed treatment with PGPR resulted in increased yield and growth in potato under field conditions (Kloepper *et al.*, 1980). van Peer and Schippers (1988) documented the increased root and shoot fresh weight of tomato, cucumber, lettuce and potato as a result of bacterization with *Pseudomonas* strains. Fluorescent pseudomonads increased the plant growth of rice and cotton by 27 and 40 per cent respectively, when the bacteria were applied to the seed (Sakthivel *et al.*, 1986).

Seeds treated with fluorescent pseudomonads resulted in increased number of tillers and grain yield in addition to control of sheath blight disease in rice (Mew and Rosales, 1986). An increase in germination of 30 to 60% in maize by plant growth promoting strains of *P. aeruginosa* strain 7NSK2 and *P. fluorescens* ANP15 was observed by Hofte *et al.* (1991). Seed and soil application of PGPR strains showed significant plant growth promotion with increased runner length and increased leaf number per plant in cucumber (Wei *et al.*, 1996). Group of *Pseudomonas* spp. significantly improved the seedling emergence in the proportion of healthy seedlings in sugar beet when compared to seedlings from untreated seeds (Williams

and Asher, 1996). Various fluorescent *Pseudomonas* strains improved vegetative sett germination, plant height, cane diameter, brix values and cane weight in sugarcane (Viswanathan and Samiyappan, 1999).

Bacterization of peanut seeds with fluorescent pseudomonads GRC_2 resulted in increased seed germination, early seedling growth, fresh nodule weight, grain yield and reduced charcoal rot disease incidence caused by *M. phaseolina* (Gupta *et al.*, 2002). Similarly, application of Pf1 as seed treatment followed by soil application against root rot effectively supported higher plant growth, better native Rhizobium nodulation and grain yield (Jayashree *et al.*, 2000).

2.8.2. Antibiosis

Many bacterial strains are known to suppress fungal growth *in vitro* by the production of one or more antifungal antibiotics that may also have activity *in vivo.* (Carruthers *et al.*, 1994; Mazzola *et al.*, 1995; Pal, 1995; Whipps, 2001). Some of these antibiotics producing strains were also shown to suppress fungal plant disease *in vitro* (Carruthers *et al.*, 1995; Leifert *et al.*, 1995; Pal, 1995). Several strains of *Pseudomonas* spp. and *Bacillus* spp. have been shown to produce wide array of antibiotics which includes ammonia, butyrolactones, 2-4 diacetyl phloroglucinol, HCN, kanosamine, oligomycin A, oomycin A, phenazine -1-carboxylic acid, pyoluteorin, pyrrolnitrin, tropolone, pyocyanin, iturin, surfactin, viscosinamide, zwittermycin A, agrocin 84 as well as several other uncharacterized moieties (Asaka and Shoda, 1996; Fujimoto *et al.*, 1995; Kerr, 1980; Maurhofer *et al.*, 1995; Mazzola *et al.*, 1995; Michereff *et al.*, 1994, Milner *et al.*, 1996; Keel and Defago, 1997; Whipps, 1997; Nielson *et al.*, 1998; Kim *et al.*, 1989; Thrane *et al.*, 1999; Nakayama *et al.*, 1999; Nowak-Thompson *et al.*, 1999).

Burkhead *et al.* (1994) reported that *P. cepacia* B37W produced pyrrolnitrin antibiotic inhibitory to *F. sambucinum.* Michereff *et al.* (1994) could correlate the *in vitro*

inhibition of *Pythium* and *Rhizoctonia* by 2, 4- diacetylphloroglucinol, an antibiotic produced by *P. fluorescens* PF5 and *in vivo* control of sorghum anthracnose caused by *Colletotrichum graminicola.*

P. fluorescens (Trevisan) Migula F113 was shown to control the potato soft rot pathogen, *Erwinia carotovora* subsp. *atroseptica* (van Hall) Dye by the production of antibiotic 2, 4-diacetylphloroglucinol (DAPG) (Cronin *et al.,* 1997). Some evidence was also obtained that siderophore production by *P. fluorescens* F113 may play a role in biocontrol of potato soft rot under iron-limiting conditions, but DAPG appears to be the major biocontrol determinant. Similar result was obtained by Khemel *et al.* (1998) that *Pseudomonas* species may also control crown gall disease in many dicotyledonous plants caused by *Agrobacterium tumefaciens.*

2.8.3. Competition

Several reports stress the importance of colonization of the biocontrol agents in rhizosphere and endorhizosphere regions of plant it protects (Benhamou *et al.*, 1996a, b, c; Forlani *et al.*, 1995; Mc lnroy and Kloepper, 1995; Quadt-Hallman *et al.*, 1997). Suppression of damping off of peas by *Burkholderia cepacia* showed a significant relationship between population size of the biocontrol agent and the degree of disease suppression (Parke, 1990). Also suppression of take all of wheat and *Fusarium* wilt of radish was correlated with colonization of roots by *Pseudomonas* strains (Bull *et al.*, 1991; Raaijmakers *et al.*, 1997).

2.8.4. Rhizosphere colonization

The crucial factor in the success of biological control by fluorescent pseudomonads is their ability to colonize the rhizosphere and their persistence throughout the growing season. Fluorescent pseudomonads are root colonizers because they occur in the natural habitat of rhizosphere and thus when they are reintroduced to roots through seed or seed-piece inoculation, they colonize root the surface profusely (van Loon *et al.*, 1998).

Scher *et al.* (1985) reported that disease suppression by fluorescent pseudomonads depends mainly on its ability to colonize rhizosphere. Introduction of *sss* gene encoding rhizosphere colonization ability into poor colonizer strain of *P. fluorescens* WCS 307 increased competitive rhizosphere colonization ability in tomato root tip resulting in increased protection against *F. oxysporum* f. sp. *radicis-lycopersici* (Dekkers *et al.*, 2000).

2.8.5. Endophytic nature

Endophytic colonization of the plant root by bacterial strain is a well known phenomenon of PGPR strains. Endophytic bacteria colonize an ecological niche similar to that of plant pathogen, especially vascular wilt pathogens, which might favour them as potential antagonistic agents. Kloepper *et al.* (1992) showed that five of six rhizobacteria which induce systemic resistance in cucumber exhibited both external and internal root colonization. Endophytic strains of *P. fluorescens* 89B-27 and *Serratia marcescens* 90-166 were observed to induce resistance in cucumber to *P. syringae* pv. *lachrymans* as well as to the fungal pathogen, *F. oxysporum* f. sp. *cucumerinum* and *C. orbiculare* (Liu *et al.*, 1995a, b). Endophytic bacteria have been shown to offer significant reduction of *F. oxysporum* f. sp. *vasinfectum* on cotton (Chen *et al.*, 1995).

Endophytic growth in roots has been recorded with PGPR *B. polymyxa* Pw-ZR and *P. fluorescens* Sm3-RN on spruce (Shishido *et al.*, 1999). Similarly, the endophytic nature of *Bacillus pumilus*, Gottheil SE34 and *P. fluorescens* 63-28 on pea (Benhamou *et al.*, 1996a, b; M'Piga *et al.*, 1997), *P. fluorescens* CHAO on tobacco (Troxler *et al.*, 1997) and *P. fluorescens* WES417r on tomato (Duijff *et al.*, 1997) has been studied intensively.

These endophytic bacteria may be in a particularly advantageous ecological position in that they may be able to grow and compete on the root surface, but also may be capable of developing within the root, relatively protected from the competitive and high-stress environment of the so. Indeed, many seeds, root and tubers are normally colonized by endophytic bacteria (McInroy and Kloepper, 1995; Struz *et al.*, 1999).

2.8.6. Induction of systemic resistance

The biocontrol agents bring about induced systemic resistance (ISR) through fortifying the physical and mechanical strength of cell wall as well as changing physiological and biochemical reaction of host leading to synthesis of defense chemicals against challenge inoculation of pathogens. Defense reaction occurs due to accumulation of PR proteins (chitinase, β-1,3-glucanase), chalcone synthase, phenylalanine ammonia lyase, peroxidase, phenolics, callose, lignin and phytoalexins.

Induced systemic resistance is a process of active resistance dependent on the host plant's physical or chemical barriers activated by biotic or abiotic agents. The ISR stimuli were shown to be salicylic acid (de Meyer and Hofte, 1997), avirulent pathogens (Kuc and Richmond, 1977) and nonpathogens such as rhizobacteria (Wei *et al.*, 1996).

Induced systemic resistance by PGPR has been achieved in large number of crops including cucumber (Wei *et al.*, 1996), tobacco (Troxler *et al.*, 1997), tomato (Duijff *et al.*, 1997), potato (Doke *et al.*, 1987), radish (Leeman *et al.*, 1996), bean (de Meyer and Hofte, 1997), sugarcane (Viswanathan and Samiyappan, 1999), chilli, brinjal (Ramamoorthy and Samiyappan, 2001) and rice (Vidhyasekaran *et al.*, 1997a, b; Nandakumar *et al.*, 2001) against broad spectrum of pathogens including fungi (Leeman *et al.*, 1995; Doke *et al.*, 1987), bacteria (Liu *et al.*, 1995a, b) and viruses (Maurhofer *et al.*, 1994; Kandan *et al.*, 2002).

In analyses of *P. aeruginosa* 7NSK2-induced resistance in bean against grey mold (de Meyer *et al.*, 1998), *P. fluorescens* WCS417-mediated protection of carnation against Fusarium wilt (van Peer *et al.*, 1991) and resistance in cucumber against anthracnose induced by six PGPR strains (Wei *et al.*, 1991) implied the involvement of ISR.

2.8.6.1. Chitinase

Chitinases (EC 3.2.1.14) are pathogenesis-related proteins (PR-proteins) which hydrolyze chitin, a major cell wall component 3-10% of higher fungi. Chitinase cleave a bond between C1 and C4 of two consecutive *N*-acetyl glucosamine (GlcNAc) either by endolytic or exolytic mechanisms. A large number of plant chitinases have been purified and characterized which are endochitinases with molecular weights ranging from 25 to 36 kDa. Many PR-proteins induced in plants treated with inducing agents have been shown to be chitinases and β-1,3-glucanases. The production of chitinases in plants has been suggested to be a part of their defense mechanism against fungal pathogens (Schlumbaum *et al.*, 1986).

In recent years, several biocontrol agents 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. to roots (Maurhofer *et al.*, 1994; Zdor and Anderson, 1992). 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 *et al.* (2001) and Nayar (1996) found early and higher induction of chitinase in *P. fluorescens* Pfl treated rice plants. Such enhanced induction of chitinase offered protection against *R. solani* in rice.

Xue *et al.* (1998) reported that non-pathogenic treatment of binucleate *Rhizoctonia* (BNR) treatment elicited a significant and systemic increase in all cellular fractions of chitinase compared to the diseased and control bean plants. Mahendran (1996) found that seed treatment with *P. fluorescens* induced chitinase isoforms with molecular weights of 31, 25 and 23 kDa in rice. Induction of four new chitinase isoforms with molecular weights of 12, 34.5, 53.5 and 63 kDa in *Pseudomonas* treated canes challenge inoculated with

C. falcatum in sugarcane was also observed (Viswanathan and Samiyappan, 2001a). Ramamoorthy *et al.* (2002a) reported the induction of 46 kDa chitinase due to *P. fluorescens* Pf1 treatment when challenge inoculated with *F. oxysporum* f. sp. *lycopersici* in tomato.

2.8.6.2. β-1,3-glucanases

Evidence of β-1,3-glucanases (E C 3.2.1.6) in disease resistance was first reported by Kauffmann *et al.* (1987). In dicots, β-1,3-glucanase genes are considered to constitute a part of the general array of defense genes induced during pathogenesis (Mauch and Staehelin, 1989). Later, induction of β-1,3-glucanases was demonstrated in barley and other monocots like wheat, rice and sorghum in response to infection by the necrotrophic pathogen, *Bipolaris sorokiniana* (Jutidamrongphan *et al.*, 1991). β-1,3-glucanases especially in conjunction with chitinase are capable of hydrolyzing fungal cell walls *in vitro* (Mauch *et al.*, 1988). Both of these enzymes are co-induced in response to fungal attack (Vogeli *et al.*, 1988).

Daugrois *et al.* (1992) reported rapid induction of two β-1,3-glucanases in the incompatible interaction between bean and *C. lindemuthianum.* Purified fungal elicitor also induced these enzymes in the bean host. Purified acidic β-1,3-glucanases from cucumber had antifungal activity against *C. orbiculare* (Ji and Kuc, 1996). Increased resistance to *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae* in transgenic tobacco expressing β-1,3-glucanase cDNA coding for the PR-N was reported by Lusso and Kuc (1996). Maurhofer *et al.* (1994) reported that *P. fluorescens* CHAO enhanced the activity of β-1,3-glucanases along with chitinases in tobacco and offered systemic protection against tobacco necrosis virus. Xue *et al.* (1998) found an 8-fold increase in β-1,3-glucanases in bean in response to BNR treatment and such treatment offered protection against pathogenic *R. solani* and *C. lindemuthianum.*

2.8.6.3. Peroxidase (PO)

Peroxidases have been implicated in the regulation of plant cell elongation, phenol oxidation, polysaccharide cross-linking, IAA oxidation, cross linking of extensin monomers, oxidation of hydroxy–cinnamyl alcohols into free radical intermediates and wound healing (Vidhyasekaran *et al.*, 1997a). Bradley *et al.* (1992) reported that the increased PO activity has been correlated with resistance in many species including barley, cucurbits, cotton, tobacco, wheat and rice and these enzymes are involved in the polymerization of proteins and lignin or suberin precursors into plant cell wall, thus constructing a physical barrier that could prevent pathogen penetration of cell walls and movement through vessels. Plant root colonization by PGPR was associated with PO activity (Albert and Anderson, 1987). Seed treatment and seedling root dipping induced early and enhanced levels of PO in rice plants (Nayar, 1996). The strains PF1 and FP7 were the best inducers of plant chitinase and peroxidases, which are the most important components of the induced systemic resistance (Nandakumar *et al.*, 2001).

Two peroxidase isoforms have been induced in the PGPR- treated rice plants inoculated with the sheath blight pathogen, *R. solani* (Nandakumar *et al.*, 2001). These enzymes are also part of the response of plant defense to pathogens (Hammerschmidt and Kuc, 1995) and they may decrease the quality of these plants as host for insects. High level expression of PO was reported in *P. fluorescens* Pf1 treated tomato plants challenged with *F. oxysporum*.f. sp. *lycopersici* (Ramamoorthy *et al.*, 2002a).

2.8.6.4. Polyphenol oxidase (PPO)

PPO usually accumulated upon wounding in plants. Biochemical approaches to understand PPO function and regulation are difficult because the quinonoid reaction products of PPO covalently modify and cross-link the enzyme. PPO can be induced *via.*, octadecanoid

defense signal pathway (Constabel *et al.*, 1995). Chen *et al.* (2000) reported that PPO was stimulated by PGPR or by the pathogen, but the wounds on split roots did not influence PPO activity compared to intact control in 13 days. PGPR untreated canes after pathogen inoculation showed comparatively lesser induction of PPO isoforms than the PGPR treated sugarcane (Viswanathan, 1999). Expression of new PPO isoform was observed in *P. fluorescens* Pf1 treated tomato plants challenged with *F. oxysporum* f. sp. *lycopersici* (Ramamoorthy *et al.*, 2002b).

2.8.6.5. Phenylalanine ammonia lyase (PAL)

PAL catalyzes the deamination of L-phenylalanine to trans-cinnamic acid, which is the first step in the biosynthesis of large class of plant natural products based on the phenylpropane skeleton, including lignin monomers as well as certain classes of phytoalexins. Induction of enzymes such as PAL and PO leading to the accumulation of phenolics and 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. In rice, ZB8 PAL gene was found to be induced by the elicitor treatment in rice cells (Li *et al.*, 1993). The gene was cloned and transgenic rice plants expressing PAL ZB8 showed systemic resistance against rice pathogens (Lamb *et al.*, 1997). Seed treatment and seedling root dip with PGPR induced early and enhanced levels of PAL in rice plants (Nayar, 1996). Plants treated with *Pseudomonas* strains initially showed higher levels of PAL compared to control (Chen *et al.*, 2000).

2.8.6.6. Strengthening of cell wall structures

The rapid strengthening of reaction sites of fungal and insect entry delays the infection process and allows sufficient time for the host to built up other defense reactions.

Seed treatment with PGPR in bean induces the lignification of cell wall (Anderson and Guerra, 1985). *Agrobacterium rhizogenes* Ri T-DNA transformed pea roots pre-inoculated with the endophytic bacterium, *Bacillus pumilus* SE34 were protected against the root rot pathogen, *F. oxysporum* f. sp. *pisi*. They found that these cell walls were strengthened at the sites of attempted fungal penetration by opposition containing large amounts of callose and phenolic substances, effectively preventing the fungal ingress. In tomato, bacterization with same bacterial strain has brought about cell wall thickening, deposition of phenolic compounds and formation of callose resulting in restricted growth of *F. oxysporum* f. sp. *radicis – lycopersici* to the epidermal cell and outer cortex in the root system in the treated plants (M'Piga *et al.*, 1997). Similar wall appositions and papillae were observed in pea roots treated with the *P. fluorescens* 63-28R upon challenge inoculation with either *F. oxysporum* f. sp. *pisi* or *P. ultimum* (Benhamou *et al.*, 1996a), indicating a general induction of physical defense barriers to pathogen ingress.

Induction of thickening of cortical cell walls in tomato was seen after colonization of roots by *P. fluorescens* WCS417 (Duijff *et al.*, 1997). *Bacillus pumilus* strain SE 34 has also induced strengthening of cell wall structure in tomato against *F. oxysporum* f. sp. *radicis-lycopersici* (Benhamou and Theriault, 1998).

2.9. Chitin as inducer of systemic resistance in plants

Involvement of chitin or chitosan in inducing systemic resistance alone or in combination with biocontrol agents has been demonstrated in few crops. Unique biological properties of chitin oligomers including their antifungal properties on various plant pathogenic fungi like *F. oxysporum* f. sp. *radicis-lycopersici* and *Pythium aphanidermatum* have been well documented (Lafontaine and Benhamou, 1996; El Ghaouth *et al.*, 1994; Leuba and Stossel, 1986). The chitin oligomers are also found as potential elicitors of plant defense reactions (Benhamou, 1992; Leuba and Stossel, 1986). Benhamou and Theriault (1998) found induction of resistance against *Fusarium* wilt by combination of chitosan with

an endophytic bacterium, *B. pumilus* strain SE 34 in tomato. Chitin amendment drastically reduced the number of stubby root nematodes (*Trichodorus* spp.) as reported by Ellis *et al.* (1998).

2.10. Neem as a biopesticide

Natural plant products and their analogues are important sources of new agricultural chemicals used for the control of plant diseases (Amadioha, 1998; Amadioha and Obi, 1998; Tewari and Nayak, 1991).

The oil and alcohol extract of *Azadirachta indica* seeds reduced rice blast (*Pyricularia oryzae*) incidence significantly *in vitro*. Apart from these, the dual culture experiment showed inhibitory nature of *A. indica* extract on the growth of *P. oryzae in vitro* (Amadioha, 2000). Solvent free EC formulation of neem oil inhibited the growth of *Magnaporthe grisea*, *Sarocladium oryzae* and *Helminthosporium oryzae* (*Cochliobolus miyabeanus*) and had no any inhibitory effect on *P. fluorescens* and *B. subtilis* (Rajappan *et al.*, 2000).

2.11. Bioformulation and method of application

Suspension from effective bacterial strains could not be used for large-scale field application due to difficulty in storage, transport and handling. In those conditions, dried commercial formulations in powder or granular forms of antagonistic bacterium forms the best alternative. Talc based formulation of *P. fluorescens* isolated from the rhizosphere of different crops has been developed (Kloepper and Schroth, 1981; Kloepper *et al.*, 1980; Vidhyasekaran and Muthamilan, 1995). PGPR could be applied in the form of bacterial suspension (Mew and Rosales, 1986; Capper and Higgins, 1993 and Thompson, 1996) and as powder formulation containing the antagonistic bacterium (Rabindran and Vidyasekaran, 1996; Vidhyasekaran *et al.*, 1997a; Nandakumar *et al.*, 2001). The methods of application include seed treatment (van Peer and Schippers 1988; Liu *et al.*, 1995a; Rosales and Mew,

1997), seedling root dip (Nayar, 1996; Maurhofer *et al.*, 1994), soil application (Samiyappan, 1988; Vidhyasekaran *et al.*, 1997b; Nandakumar *et al.*, 2001) and foliar application (Mew and Rosales, 1986; Chatterjee *et al.*, 1996). Combination of individual treatments can also be practiced (Rabindran and Vidyasekaran, 1996; Nandakumar, 1998). Nandakumar *et al.* (2001) and Radja Commare *et al.* (2002) observed significant reduction in sheath blight incidence and leaf folder attack in rice when *P. fluorescens* strain mixtures were applied as seed treatment followed by soil application and foliar spray.

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

3.1. Plant materials and pathogens

Tea plants, from tea plantations (48 years old) of EID Parry Agro-industries Pvt. Ltd. Anamalai hills, Valparai, Coimbatore district were used to study the efficacy of plant growth promoting rhizobacteria (PGPR) strains against the biotroph pathogen (*Exobasidium vexans*) causing blister blight disease.

Seeds of green gram *cv.* Co6 were used to test the efficacy of PGPR strains against the necrotroph pathogen (*Macrophomina phaseolina*) causing root rot disease. Seeds were obtained from Pulses Breeding Station, Tamil Nadu Agricultural University, Coimbatore.

3.1.1. Isolation of pathogen and pathogenicity test

The plants infected with *M. phaseolina* showing typical root rot symptoms were collected and pathogen was isolated using potato dextrose agar (PDA) medium. Pure cultures of the pathogen was obtained by single hyphal tip method (Rangaswami, 1972). Sand-maize medium containing sand and maize @ 19:1 respectively was sterilized at 121°C, 15psi for 2 hrs. The isolates were inoculated into sand-maize medium and incubated for 15 days at room temperature 28±5°C for multiplication (Riker and Riker, 1936). Potting soil (redsoil : sand : cowdung manure @ 1:1:1 w/w/w) was autoclave sterilized at 121°C, 15 psi for two hours in two consecutive days. Pathogenicity of the isolates were tested by artificially inoculating the sand-maize culture in the potting soil at 10% w/w. The potting soil incorporated with the fungus was filled in 30 cm diameter

pots. Seeds of green gram were surface sterilized with 0.1 per cent mercuric chloride for 30 seconds and rinsed three times with sterile distilled water and sown @ 20 seeds per pot separately. The pots were watered at weekly intervals and observations were made on 30 days after sowing.

3.2. Isolation and maintenance of biocontrol agents

Soil samples were collected from the rhizosphere region of tea (*cv.* Assam) plants grown at Valparai area of Anamalai hills. The PGPR strains *viz.*, *Pseudomonas* spp., *Bacillus spp.*, were isolated from the soil samples by using the serial dilution technique in Kings' B (KB) and Nutrient Agar (NA) medium respectively (Annexure I & II). The cultures of antagonistic microorganisms were identified based on their morphological characters.

Pseudomonas fluorescens (Pf-1) culture was obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. Pure cultures of *P. fluorescens* and *B. subtilis* were maintained on KB and NA slants respectively at 4 °C for further studies.

3.3. *In vitro* testing of PGPR strains on inhibition of mycelial growth of *M. phaseolina*

PGPR strains were tested for their inhibition on mycelial growth of *M. phaseolina* by following the dual culture technique (Dennis and Webster, 1971). The bacterial culture was streaked at one side of Petri dish (1 cm from the edge of the plate) plated with PDA medium and mycelial disc (8 mm diameter) of seven days old culture of *M. phaseolina* was placed on the opposite side in the Petri dish perpendicular to the bacterial streak. The plates were incubated at room temperature (28±2°C) for four days. The mycelial inhibition of pathogen was measured in millimetre.

3. 4. Efficacy of PGPR strains on plant growth under laboratory conditions

3. 4. 1. Preparation of bacterial inoculum

P. fluorescens and *B. subtilis* were grown in conical flasks (250 ml) containing 100 ml of KMB broth and NA broth respectively for 48 h on a rotary shaker (150 rev min^{-1}) at 28±2°C. Cells were removed by centrifugation at 8000 rpm for 10 min at 4°C and washed in sterile water. The pellet was resuspended in small quantity of sterile distilled water and then diluted with adequate amount of sterile distilled water.

3.4.2. Seed bacterization

Seeds of green gram (*cv.* Co6) were surface sterilized with two per cent sodium hypochlorite for 30 sec, rinsed in sterile distilled water and dried overnight under sterile air stream. PGPR strains inoculated into respective broth was taken in a Petri dish. To this, 100 mg of carboxymethylcellulose (CMC) was added as an adhesive material. One gram of seeds were soaked in 10 ml of bacterial suspension (containing $3x10^8$ cfu/ml) for 2 h and dried overnight in a sterile Petri dish.

3.4.3. Plant growth-promotion

Plant growth-promoting activity of PGPR strains were assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993). Seed bacterization was done as described in 3.4.2. Twenty seeds were kept over the presoaked germination paper. The seeds were held in position by placing another presoaked germination paper strip and gently pressed. The polythene sheet along with seeds were then rolled and incubated in growth chamber for 15 days. Three replications were maintained for each treatment. The root length and shoot length of individual seedlings were measured and the germination percentage of seeds was also calculated.

The vigour index was calculated by using the formula as described by Abdul Baki and Anderson (1973).

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

3.5. Preparation of talc based formulations of PGPR strains

3.5.1. Talc-based formulation for individual strain

A loopful of *P. fluorescens* was inoculated into the King's B broth and incubated in a rotary shaker at 150 rpm for 72 h at room temperature (28 ± 2°C). After 72 h of incubation, the broth containing 9x 10^8 cfu/ml was used for the preparation of talc-based formulation. To the 400 ml of bacterial suspension, 1 kg of the purified talc powder (sterilized at 105° C for 12 h), calcium carbonate 15 g (to adjust the pH to neutral) and carboxymethyl cellulose (CMC) 10 g (adhesive) were mixed under sterile conditions, following the method described by Vidhyasekaran and Muthamilan (1995). The product was shade dried to reduce the moisture content below 20 per cent and then packed in polypropylene bag and sealed. At the time of application, the population of bacterium in talc formulation was checked to 2.5 to 3 x 10^8 cfu/g.

The formulation of *B. subtilis* was developed by the same procedure as mentioned above using Nutrient broth medium.

3.5.2. Talc based formulation for *Pseudomonas* and *Bacillus* mixtures

The two PGPR bacteria *viz., P. fluorescens* and *B. subtilis* were grown in respective broth separately and the two bacteria which are going to make up the mixture were added equally (v/v) and finally mixed with talc powder, CaCO3 and CMC as described in 3.5.1.

3.5.3. Chitin amendment with talc-based formulations

3.5.3.1. Preparation of colloidal chitin

Five g of crab shell chitin (Sigma, USA) was slowly added into 100 ml of cold 0.25 N HCl with vigorous stirring and kept overnight at 4°C. The mixture was filtered through glasswool into 200 ml of ice cold ethanol at 4°C with rapid stirring. The resultant chitin suspension was centrifuged at 10000 rpm for 20 min and the chitin pellets were washed repeatedly with distilled water until pH becomes neutral. The concentration of colloidal chitin was adjusted to 10 mg per ml.

3.5.3.2. Preparation of talc-based formulations amended with chitin

Colloidal chitin was incorporated into KB and NA broth @ 1%v/v. After sterilization, the bacterial cultures, *P. fluorescens* and *B. subtilis* were inoculated in KB broth and NA broth respectively and talc based formulations were prepared as mentioned in 3.5. 1.

3.5.4. Neem amendments in talc-based formulation

3.5.4.1. Preparation of neem seed kernel extract

Seeds from mature dehisced fruits of *Azadirachta indica* were oven dried for 2 days at 60°C. The seeds were ground using liquid nitrogen in pestle and mortar. The powdered neem was extracted with cold water (Amadioha, 2000).

3.5.4.2. Preparation of talc-based formulations amended with neem

One ml of neem seed kernel extract was added into 100 ml of King's B broth and Nutrient broth. After sterilization, *P. fluorescens* (Pf1) and *B. subtilis* were inoculated into their respective neem based media, incubated in shaker and the formulations were prepared as described in 3.5.1.

3.5.5. Preparation of talc-based formulations amended with both neem and chitin

3.5.5.1. Preparation of talc-based formulations of individual strains amended with neem and chitin

Colloidal chitin and neem kernal extract were added @ 1% (v/v) into the KB and NA broth. After sterilization, *P. fluorescens* and *B. subtilis* were inoculated into respective broth and formulations were prepared as described in 3.5.1.

3.5.5.2. Preparation of talc-based formulations of mixed strains amended with both neem and chitin

P. fluorescens and *B. subtilis* were grown on their respective broth separately after the amendment of Neem and chitin. After incubation, the broth culture was added equally (v/v) and finally mixed with talc powder, CaCO3 and CMC and shade dried.

3.6. Method of application of bioformulations and chemicals

3.6.1. Application of bioformulations and chemicals against root rot of green gram

3.6.1.1. Seed treatment

Seeds were soaked in double the volume of sterile distilled water containing talc-based formulation (10 g/kg of seed). After 24 h, the suspension was drained off and the seeds were dried under shade for 30 min and used for sowing (Vidhyasekaran *et al.*, 1997a). Carbendazim @ 2g/kg of seed was applied as chemical control.

3.6.1.2. Soil application

Five g of talc-based formulation per kg of soil was added during sowing and planting. In field, biocontrol agents were applied @ 2.5 kg/ha on 30 days after sowing

(DAS). Carbendazim @ 0.1% was applied as soil-drench to pot culture and field experiments.

3.6.2. Application of bioformulation and chemical against blister blight of tea

Talc based formulation @ 0.5% and Hexaconazole @ 0.25 % were given as foliar spray. Hexaconazole as chemical check was used to have the comparative study with the biocontrol agents.

3.7. Efficacy of PGPR formulations against root rot of green gram under greenhouse conditions

The virulent isolate of *M. phaseolina,* mass multiplied in the sand-maize medium was mixed with the sterilized potting soil. Surface sterilized green gram seeds treated with different combinations of PGPR formulations @ 10g/kg of seeds were sown in 30 diameter pots @ 20 seeds per pot. The treatments and design of pot culture experiments were as follows.

Treatments:

T_1: *P. fluorescens*

T_2: *P. fluorescens* + Chitin

T_3: *P. fluorescens* + neem

T_4: *P. fluorescens* + chitin + neem

T_5: *B. subtilis*

T_6: *B. subtilis* + chitin

T_7: *B. subtilis* + neem

T_8: *B. subtilis* + chitin + neem

T_9: *P. fluorescens* + *B. subtilis*

T_{10}: *P. fluorescens* + *B. subtilis* + chitin

T_{11}: *P. fluorescens* + *B. subtilis* + neem

T_{12}: *P. fluorescens* + *B. subtilis* + chitin + neem

T_{13}: Carbendazim

T_{14}: Control

Design : Completely Randomized Block Design (CRD)

Replication : Two

Observation : 30, 45 and 60 DAS

3.7.1. Disease assessment

The per cent disease incidence (PI) was assessed using the following formula

$$PI = \frac{\text{Number of infected plants}}{\text{Total number of seeds sown}} \times 100$$

3.7.2. Yield assessment

The yield attributing parameters *viz.*, number of pods/ plant, number of seeds/ pod, root nodulations, root length and shoot length were recorded. Pulse grain yield was also recorded after harvesting the crop.

3.8. Efficacy of PGPR formulations against root rot of green gram under field conditions

A field trial was conducted at Pulse Breeding Station, Tamil Nadu Agricultural University, Coimbatore. The package of practice was followed from standard Crop Production Manual 2000.

The treatment details were as follows

Treatments:

T_1: *P. fluorescens*

T_2: *P. fluorescens* + Chitin

T_3: *P. fluorescens* + neem

T_4: *P. fluorescens* + chitin + neem

T_5: Carbendazim

T_6: Control

Design : Randomized Block Design (RBD)

Replication : Three

Plot size : 4m x 3m

Spacing : 30cm x 10cm

Observation : 30, 45 and 60 DAS

Season : Kharif, 2002

3.8.1. Disease assessment

From each plot, 1 m^2 area was selected randomly from three locations. Total number of plants and infected plants were taken into account and the per cent disease incidence (PI) was calculated using the following formula

$$PI = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

3.8.2. Yield assessment in green gram

The yield attributing parameters *viz.*, number of pods/ plant, number of seeds/ pod were recorded. The grain yield per ha was calculated.

3.9. Efficacy of PGPR bioformulations against blister blight of tea under field conditions

A field trial was conducted at Iyerpadi estate of Parry Agro-industries Pvt Ltd., Valparai, Coimbatore District. The disease incidence was taken at 30 days after first spray. The different treatment details were as follows

Treatments:

T_1: *P. fluorescens* at 7 days interval

T_2: *P. fluorescens* at 14 days interval

T_3: *P. fluorescens* at 21 days interval

T_4: *B. subtilis* at 7 days interval

T_5: *B. subtilis* at 14 days interval

T_6: *B. subtilis* at 21 days interval

T_7: *P. fluorescens* + *B. subtilis* + Chitin + neem at 7 days interval

T_8: *P. fluorescens* + *B. subtilis* + Chitin + neem at 14 days interval

T_9: *P. fluorescens* + *B. subtilis* + Chitin + neem at 21 days interval

T_{10}: Hexaconazole spray at 5 days interval

T_{11}: Untreated control

Design : Randomized block design (RBD)

Replication : Three

Plot size : 100 bushes / plot (8mx8m)

Duration :2001 and 2002. (Pruning was done during 15th July of 2002).

Observation : Every 15 days interval

3.9.1. Disease assessment for blister blight of tea

Fifty leaves were collected randomly from each plot and they were assessed for the presence of blistered spots. The per cent disease index (PDI) was calculated following disease scoring scale (Plate 1).

Category	Grade
No disease	0
1 % of leaf area affected	1
1-10 % of leaf area affected	3
11-25 % of leaf area affected	5
25 – 50 % of leaf area affected	7
> 50 % leaf area affected	9

Plate 1. Grading of disease incidence in tea plantations

Category	Grade
No disease	0
1 % of leaf area affected	1
1-10 % of leaf area affected	3
11-25 % of leaf area affected	5
25 – 50 % of leaf area affected	7
> 50 % leaf area affected	9

Plate 1

$$PDI = \frac{\text{Sum of all individual ratings}}{\text{Total number of leaves}} \times \frac{100}{\text{Maximum disease grade}}$$

3.9.2. Yield assessment in tea

Plucking of three top leaves along with young shoots was done every fifteen days interval. Yield data per plot was recorded regularly during the course of study period and yield was correlated with disease incidence.

3.10. Analysis of defense related proteins and chemicals against root rot of green gram and blister blight of tea

3.10.1. Collection of plant samples

Green gram root samples were collected at 7 days interval starting from zero day to 49 days after inoculation of pathogen and pre-treated with different bioformulation combinations. Four plants were sampled from each replication of the treatment separately.

3.10.1.1. Enzyme extraction

One g of root sample was homogenized with 2 ml of 0.1M sodium citrate buffer (pH 5.0) at 4°C. The homogenate was centrifuged for 20 min. at 10000 rpm. The supernatant was used as crude enzyme extract for assaying chitinase activity. Enzyme extracted in 0.1 M sodium phosphate buffer (pH 7.0) was used for the estimation of Peroxidase (PO), Polyphenol Oxidase (PPO) and Phenylalanine Ammonia-Lyase (PAL). Enzyme extract was stored in deep freezer (-70° C) until used for biochemical analysis.

Protein content in the extracts were determined by the method of Bradford (1976). Ten milligram 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 upto 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 GS5703 AT spectrophotometer. Bovine serum albumin was used as the standard.

For tea blister blight, leaf samples were collected from field treated with different bioformulations. Enzyme extraction was prepared and protein content was measured as mentioned earlier.

3.10.2. Phenyl alanine ammonia lyase (PAL)

The PAL assay was carried out as per the method described by Ross and Sederoff (1992). The assay mixture containing 100 µl of enzyme, 500 µl of 50 mM Tris HCl (pH 8.8) and 600 µl of 1 mM L-phenylalanine was incubated for 60 min and the reaction was arrested by adding 2 N HCl. Later 1.5 ml of toluene was added, vortexed for 30 sec, centrifuged (1000 rpm, 5 min) and toluene fraction containing trans-cinnamic acid was separated. The toluene phase was measured at 290 nm against the blank of toluene. Standard curve was drawn with graded amounts of cinnamic acid in toluene as described earlier. The enzyme activity was expressed as nmoles of cinnamic acid min^{-1} mg^{-1} of protein.

3.10.3. Peroxidase (PO)

Assay of PO activity was carried out as per the procedure described by Hammerschmidt *et al.* (1982). The reaction mixture consisted of 2.5 ml of a mixture containing 0.25 per cent (v/v) guaiacol in 0.01 M sodium phosphate buffer, pH 6.0 and 0.1 M hydrogen peroxide. Enzyme extract (0.1ml) was added to initiate the reaction, which

was followed colorimetrically at 470 nm. Crude enzyme preparations were diluted to give changes in absorbance at 470 nm of 0.1 to 0.2 absorbance units/min. The boiled enzyme preparation served as blank. Activity was expressed as the increase in absorbance at 470 nm min^{-1} mg^{-1} of protein.

3.10.4. Polyphenol oxidase (PPO)

PPO activity was determined as per the procedure given by Mayer *et al.* (1965). The reaction mixture consisted of 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. To start the reaction, 0.01 M catechol was added and the activity was expressed as changes in absorbance (in units) min^{-1} mg^{-1} of protein.

3.10.5. Phenolics content

Phenol content of green gram and tea was estimated as per the procedure given by Zieslin and Ben-Zaken (1993). One gram of tissue was homogenized in 10 ml of 80% methanol and agitated for 15 minutes at 70 °C. One ml of the methanol extract was added to 5 ml of distilled water and 250 µl of Folin Ciocalteau reagent (1N) and the solution was kept at 25 °C. After three min. one millilitre of saturated solution of Na_2CO_3 and one ml of distilled water was added and the reaction mixture was incubated for 1 h at 25 °C. The absorption of the developed blue colour was measured using a GS 5703 AT spectrophotometer at 725 nm. The content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Ciocalteau reagent with a phenol solution (C_6H_5OH) and expressed as catechol equivalents mg^{-1} of protein.

3.10.6. Chitinase

The colorimetric assay of chitinase was carried out as per Boller and Mauch (1988). Reagents used were colloidal chitin, snail gut enzyme, dimethyl amino benzaldehyde (DMAB) and buffer (Annexure III)

3.10.6.1. Assay procedure

The reaction mixture consisted of 10 µl of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme solution and 0.1 ml colloidal chitin (10 mg). After incubation for 2 h at 37 °C, the reaction was stopped by centrifugation at 1000 rpm for 3 mın. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl of 1 M potassium phosphate buffer (pH 7.0) and incubated with 20 µl of 3 % (w/v) snail gut enzyme for 1 h. After 1 h, the reaction mixture was brought to pH 8.9 by the addition of 70 µl of 0.1 M 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, the mixture was incubated for 20 min at 37°C and immediately thereafter the absorbance was measured at 585 nm. *N*-acetylglucosamine (GlcNac) was used as a standard. The enzyme activity was expressed as nmoles GlcNac equivalents min^{-1} mg^{-1} of protein.

3.10.7. *β*-1,3-glucanase

β-1,3-glucanase activity was assayed by the laminarin dinitrosalicylic acid method (Pan *et al.*, 1991). The reaction mixture consisted of 62.5 µL of 4% laminarin and 62.5 µl of enzyme extract. The reaction was carried out at 40 °C for 10 min The reaction was then stopped by adding 375 µL of dinitrosalicylic acid and heated for 5 min in a

boiling water, vortexed and the absorbance was measured at 500 nM. The enzyme activity was expressed as µg glucose released $min^{-1}mg^{-1}$protein.

3.10.8. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

One gram of powdered root sample was extracted with one millilitre of 0.1M sodium phosphate buffer (pH 7.0) under 4°C. The homogenate was centrifuged for 20 min. at 10000 rpm. and the supernatant was used for the SDS-PAGE (Laemmli, 1970). The protein content of the sample was determined by Bradford method (Bradford, 1976).

Hundred microgram of protein from different treatments was taken and mixed with 10 µl of sample buffer in a microfuge tube, boiled for 4 min and incubated at 4°C for 30 min. Then the samples containing equal amount of proteins were loaded into the wells of polyacrylamide gels (Annexure IV) (Sigma-Aldrich Techware system, Sigma, USA). The medium range molecular weight markers (Bangalore Genei, India) were used and electrophoresis was carried out at constant voltage of 75 volts for 2 h. The gels were stained with 0.2 % Coomassie brilliant blue (R250) solution. Based on the Rf value of each protein band stained, the molecular weight was calculated.

3.10.9. Western blotting

After SDS-PAGE, the proteins were electroblotted onto 0.45 µm PVDF (Millipore) membranes (Sigma, USA) as per Gallagher *et al.* (1995). The electrophoretic transfer of proteins was carried out from gel to membrane in a Bio Rad semidry transblot apparatus (9140 mA, 30 min). The membranes were then stained with Ponceau S stain (Sigma, USA) for 2 min to check the resolution and transfer quality. Ponceau S stain was destained with TBST for 2 min and the membrane was blocked for 1.5 h at room

temperature (28 ± 2 °C) in TBST containing 2.5 % (w/v) gelatin. The membrane was then soaked in the diluted primary antibody (barley chitinase), a gift from Dr. S. Muthukrishanan, Kansas State University, Manhattan, Kansas 66506, USA (1:3000 dilution) overnight in TBST. After incubating with the primary antibody, the membrane was washed with TBST thrice for 10 to 15 min each time to remove the unbound antibody. The membrane was then incubated in secondary antibody for 3 h affinity purified goat anti-rabbit immunoglobulin (IgG) alkaline phosphatase conjugate (Sigma, USA) was used as secondary antibody (1:7000). The membrane was then washed thrice with TBST and thrice in TBS for 10 to 15 min each time. Immunological reaction was visualized by soaking the membranes in alkaline phosphatase colour development reagents (Bangalore Genei, India). Immediately after colour development, the membranes were washed in distilled water and dried (Annexure V).

3.10.10. Native gel electrophoresis

3.10.10.1. Peroxidase (PO)

To study the expression pattern of different isoforms of peroxidases in different treatments, activity gel electrophoresis was carried out for native anionic polyacrylamide gel electrophoresis, resolving gel of 8% acrylamide concentration and stacking gel of 4% acrylamide concentration were prepared (Annexure VI). After electrophoresis, the gels were incubated in the solution containing 0.15% benzidine in 6% NH_4Cl for 30 min in dark. Then few drops of 30% H_2O_2 were added with constant shaking till the bands appear. After staining, the gel was washed with distilled water and photographed (Sindhu *et al.*, 1984).

3.10.10.2. Polyphenol oxidase (PPO)

After native electrophoresis, the gel was equilibrated for 30 min in 0.1% *ρ*-phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer (Jayaraman *et al.*, 1984). The addition of catechol was followed by a gentle shaking which resulted in appearance of dark brown discrete protein bands.

3.11. Statistical analysis

The data were statistically analyzed (Gomez and Gomez, 1984) and treatment means were compared by Duncan's Multiple Range Test (DMRT). The package used for analysis was IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines.

Experimental Results

CHAPTER IV
EXPERIMENTAL RESULTS

PGPR bioformulations were tested against the blister blight and dry root rot disease in tea and green gram. The following results were obtained during the course of experiment.

4.1. Isolation of pathogen and pathogenicity test

Healthy green gram seeds sown in the soil were artificially inoculated with the *Macrophomina phaseolina*, dry root rot pathogen produced the typical root rot symptoms both in root and stem on 25 days after sowing. The pathogen was reisolated and inoculated into the soil where seeds of green gram were sown. Typical root rot symptoms were observed from the seedlings of green gram and Koch's postulates were proved.

4.2. Efficacy of PGPR strains on plant growth promotion

Green gram seeds treated with the different bacterial suspensions showed improvement in plant growth parameters over untreated seeds. *Pseudomonas fluorescens* isolates (Pf1, PfG2 and PfV3) and *Bacillus subtilis* were found to increase the vigor index of green gram seedlings significantly. The increase in mean root length (12.3 cm) and shoot length (11.5 cm) due to Pf1 was significantly higher compared to the seedlings from untreated control (Plate 2). The maximum vigor index of 2372 was observed in green gram seedlings treated with Pf1 suspension and less vigor index of 1191 was recorded from untreated control (Table 1).

4.3. *In vitro* screening of the PGPR strains against the pathogen

Different strains of PGPR were tested against the mycelial growth of *M phaseolina*. Among the different PGPR strains, *P. fluorescens* Pf1 showed the highest inhibition of *M. phaseolina* followed by *B. subtilis*. The per cent inhibition was significantly higher in case of Pf1 (44.55) whereas in case of control the full growth in the plate of *M. phaseolina* was recorded (Table 2) (Plate 3).

Table 1. Effect of seed treatment with PGPR strains on growth attributes of green gram

Treatments	MSL (cm)	MRL (cm)	Germination (%)	Vigour index
P. fluorescens (Pf1)	12.3[d]	11.5[d]	99.7[d] (86.99)	2372.86[d]
Pf G1 (green gram isolate)	8.1[b]	7.6[b]	93.7[b] (75.46)	1471.09[b]
Pf V1 (Valparai isolate)	8.2[b]	7.8[b]	93.0[b] (74.65)	1488.00[b]
B. subtilis (Bs1)	10.2[c]	9.4[c]	96.7[c] (79.53)	1895.32[c]
Control	7.2[a]	6.5[a]	87.0[a] (68.58)	1191.9[a]

MSL – Mean shoot length

MRL - Mean root length

Values in parentheses are arcsine transformed.

In a column, means followed by a common letter are not significantly different (p=0.05) by DMRT

Plate 2. Effect of *Pseudomonas fluorescens* strain on seed germination and seedling vigour in green gram seedlings

Plate 2

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Table 2. Efficacy of PGPR strains in inhibiting mycelial growth of *M. phaseolina*

Treatments	Mycelial growth of pathogen (mm)	Per cent inhibition
P. fluorescens (Pf1)	49.9	44.55[e]
Pf G1 (green gram isolate)	81.5	9.44[c]
Pf V1 (Valparai isolate)	82.4	8.44[b]
B. subtilis	79.5	16.11[d]
Control	90.0	0.00[a]

Values are mean of four replications.

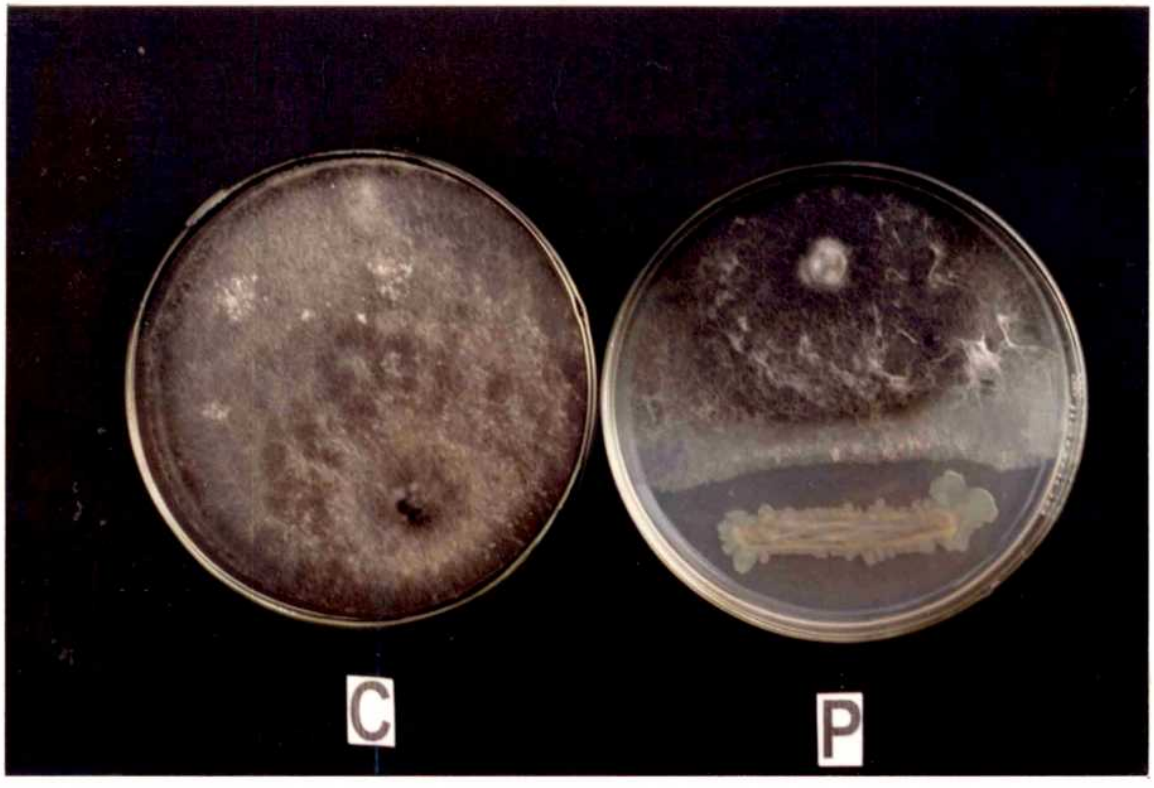
In a column, means followed by a common letter are not significantly different (p=0.05) by DMRT

Plate 3. Dual culture technique for the effect of *P. fluorescens* on mycelial growth of *M. phaseolina*

C- Control

P- *P. fluorescens*

Plate 3



4.4. Efficacy of PGPR formulations against root rot disease of green gram

4.4.1. Greenhouse experiments

4.4.1.1. Efficacy of PGPR formulations against root rot incidence

The PGPR strains which showed significantly higher vigour index and inhibition over the mycelial growth of *M. phaseolina* were selected and screened against dry root rot disease under greenhouse conditions. Among the different combinations of bioformulations used, application of *P. fluorescens* Pf1 along with chitin as seed treatment and soil application resulted in less root rot incidence of 0.0, 2.5 and 7.5 per cent at 30, 45 and 60 days after sowing (DAS) respectively which showed significant different from the untreated control plants (Plate 4). The per cent reduction in disease incidence by *P. fluorescens* Pf1 was comparable with that of chemical treatment (Table 3).

4.4.1.2. Effect of PGPR strains on yield of green gram

The biometrical observations were taken at 30, 45 and 60 days after sowing. *P. fluorescens* Pf1 combination with chitin showed significantly higher root and shoot length throughout the study period (Table 4) (Plate 5). The same trend was observed in case of number of seeds per pod and number of pods per plant also. Significantly higher yield was recorded in *P. fluorescens* Pf1 amended with chitin (9.25g/pl.) bioformulation followed by Pf1 combination with chitin and neem (7.10g/pl.). Untreated control recorded the lowest yield of 3.06g/pl. In general all the treatments were effective in increasing the yield when compared to untreated control (Table 5).

4.4.2. PGPR induced defense related protein and chemicals against the root rot pathogen

4.4.2.1. Peroxidase (PO)

Peroxidase activity was measured in roots from the *M. phaseolina* inoculated and PGPR bioformulations pretreated plants. The induction of PO activity in green gram was observed at 7 days after challenge inoculation with *M. phaseolina* pretreated with Pf1

Table 3. Efficacy of PGPR strains on root rot incidence of green gram under greenhouse conditions

Treatments	30 DAS	45 DAS	60 DAS	Mean	Per cent reduction over control
Pf1	2.5^{c} (9.05)	7.5^{e} (15.88)	12.5^{e} (20.70)	7.5^{e}	74.20
Pf1 + Chitin (C)	0.0^{e} (0.00)	5.0^{f} (12.90)	7.5^{g} (15.88)	4.1^{f}	85.62
Pf1 + Neem (N)	2.5^{c} (9.05)	12.5^{c} (20.70)	17.5^{c} (24.72)	10.8^{c}	62.78
Pf1 + C + N	2.5^{c} (9.05)	10.0^{d} (18.42)	10.0^{f} (18.42)	7.5^{d}	74.20
Bacillus (B)	5.0^{b} (12.90)	10.0^{d} (18.42)	20.0^{bc} (26.56)	11.6^{d}	59.92
B + C	2.5^{c} (9.05)	7.5^{e} (15.88)	17.5^{c} (24.72)	9.1^{e}	68.49
B + N	5.0^{b} (12.90)	15.0^{b} (22.78)	22.5^{b} (28.31)	14.1^{b}	51.36
B + N + C	2.5^{c} (9.05)	10.0^{d} (18.42)	20.0^{bc} (26.56)	10.8^{d}	62.78
P + B	2.5^{c} (9.05)	10.0^{d} (18.42)	15.0^{d} (22.78)	9.1^{d}	68.49
P + B + C	0.0^{e} (0.00)	7.5^{e} (15.88)	12.5^{e} (20.70)	6.6^{e}	77.05
P + B + N	5.0^{b} (12.90)	15.0^{b} (22.78)	20.0^{bc} (26.56)	13.3^{b}	54.21
P+ B + N + C	2.5^{c} (9.05)	10.0^{d} (18.42)	15.0^{d} (22.78)	9.1^{d}	68.49
Carbendazim	0.0^{d} (0.00)	5.0^{f} (12.90)	7.5^{g} (15.88)	4.1^{f}	85.62
Control	15.0^{a} (22.78)	25.0^{a} (29.99)	47.5^{a} (43.56)	29.1^{a}	0.00

DAS – Days After Sowing

Values in parentheses are arcsine transformed

In a column, means followed by a common letter are not significantly different (p=0.05) by DMRT

Plate 4. Effect of *P. fluorescens* along with chitin bioformulation on root rot incidence of green gram under greenhouse conditions

1- Control

2. *P. fluorescens* + Chitin

Plate 4

Table 4. Effect of PGPR strains on plant biometrics of green gram under greenhouse conditions

Treatments	30 DAS		45 DAS		60 DAS	
	Root length (cm)	Shoot length (cm)	Root length (cm)	Shoot length (cm)	Root length (cm)	Shoot length (cm)
Pfl (P)	13.4^{cd}	38.7^{cde}	15.1^{d}	48.1^{ef}	18.2^{d}	55.2^{ef}
Pfl + Chitin (C)	16.2^{a}	42.3^{a}	18.4^{a}	53.9^{a}	22.8^{a}	67.9^{a}
Pfl + Neem (N)	12.5^{e}	36.8^{def}	15.2^{d}	47.1^{cd}	18.2^{d}	54.6^{ef}
Pfl + C + N	15.7^{a}	40.2^{abc}	17.5^{b}	49.9^{b}	20.3^{b}	60.2^{bc}
Bacillus (B)	12.3^{e}	35.1^{f}	14.2^{e}	43.1^{ef}	18.2^{d}	53.1^{f}
B + C	13.5^{c}	37.4^{c-f}	15.1^{d}	46.8^{d}	19.1^{c}	55.2^{ef}
B + N	10.9^{f}	29.1^{gh}	13.2^{f}	42.8^{ef}	16.1^{f}	49.3^{g}
B + N + C	12.8^{de}	36.1^{ef}	15.4^{d}	43.7^{e}	18.2^{d}	$54\ 1^{ef}$
P + B	13.6^{bc}	39.1^{bcd}	15.4^{d}	47.7^{bcd}	18.2^{d}	56.2^{de}
P + B + C	14.2^{b}	41.8^{ab}	16.1^{c}	49.8^{bc}	19.9^{b}	61.2^{b}
P + B + N	12.4^{e}	37.9^{bcd}	14.1^{e}	46.3^{d}	17.9^{d}	55.2^{ef}
P+ B + N + C	13.9^{bc}	39.2^{bcd}	15.2^{d}	48.6^{bcd}	18.5^{cd}	58.3^{cd}
Carbendazim	11.24^{f}	31.4^{g}	13.2^{f}	40.7^{f}	16.8^{e}	52.6^{f}
Control	8.5^{g}	26.8^{h}	11.0^{g}	35.1^{g}	14.0^{g}	46.9^{g}

Values are mean of two replications

In a column, means followed by the same letter are not differ significantly (p=0.05) by DMRT

Plate 5. Effect of *P. fluorescens* amended with chitin bioformulation on growth and vigour of green gram seedlings under greenhouse conditions

Plate 5

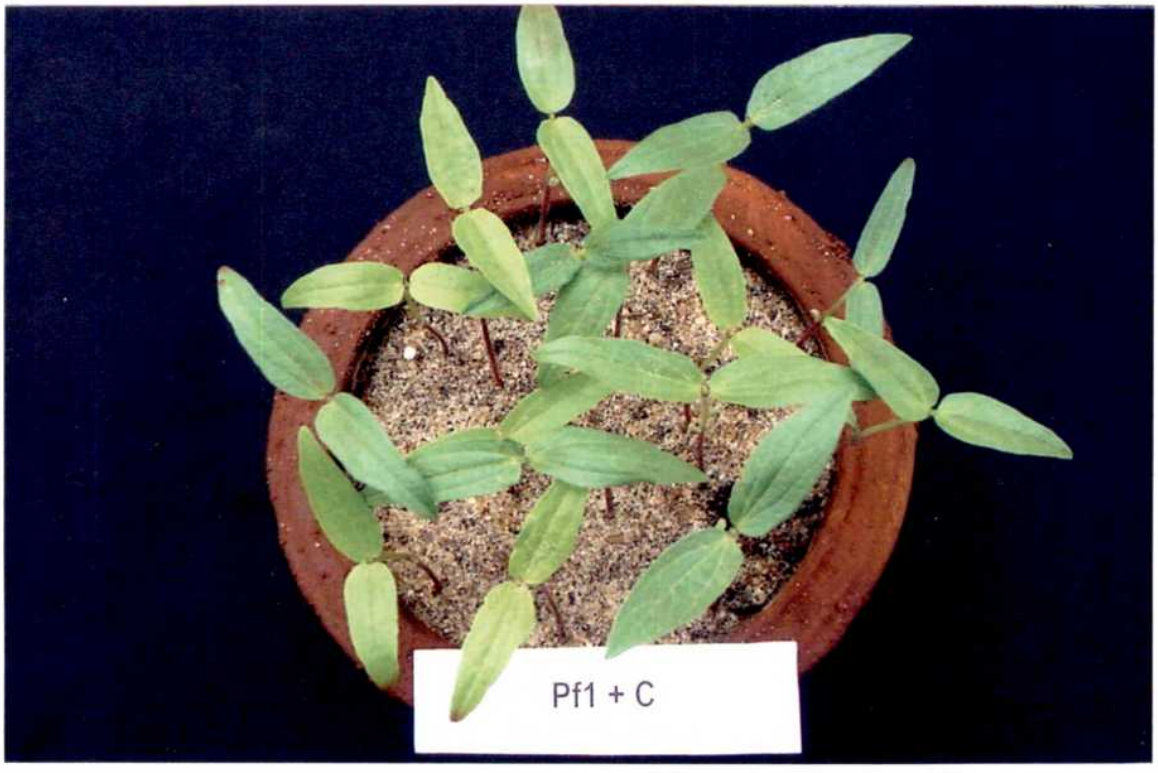


Table 5. Efficacy of PGPR strains on yield of green gram under greenhouse conditions

Treatments	Number of seeds / pod	Number of pods / plant	Grain yield (g/plant)	Per cent increase over control
Pf1 (P)	12.5^{bc}	14.5^{cd}	6.25^{gh}	104.24
Pf1 + Chitin (C)	15.5^{d}	16.5^{e}	9.23^{J}	201.63
Pf1 + Neem (N)	10.0^{c}	13.0^{bc}	4.20^{b}	37.25
Pf1 + C + N	13.5^{b}	15.0^{de}	7.10^{I}	132.02
Bacillus (B)	12.5^{bc}	12.5^{b}	5.25^{de}	71.56
B + C	12.0^{bc}	13.5^{bc}	5.48^{def}	79.08
B + N	11.5^{c}	12.0^{bc}	4.52^{bc}	47.71
B + N + C	12.0^{b}	13.0^{b}	5.24^{def}	71.24
P + B	12.5^{bc}	13.5^{bc}	5.75^{efg}	87.90
P + B + C	13.0^{c}	14.5^{cd}	6.54^{hi}	113.72
P + B + N	11.0^{b}	12.0^{b}	4.28^{b}	39.86
P+ B + N + C	12.5^{bc}	14.0^{cd}	6.00^{fgh}	96.07
Carbendazim	12.5^{bc}	12.0^{b}	5.00^{cd}	63.39
Control	9.0^{a}	8.5^{a}	3.06^{a}	0.00

Values in parentheses are arcsine transformed

In a column, means followed by a common letter are not significantly different (p=0.05) by DMRT

amended chitin bioformulation. The enzyme activity was significantly increased upto 21 days after challenge inoculation and the maximum induction was observed during this period. The enzyme activity declined at 35 days after challenge inoculation where in untreated control, the enzyme activity drastically reduced 21 days after challenge inoculation (Table 6) (Fig. 1).

4.4.2.2. Polyphenol oxidase (PPO)

Upon pathogen inoculation, PPO activity also significantly increased in *P. fluorescens* Pf1 amended with chitin bioformulation treated green gram roots than the inoculated control. PPO accumulation reached maximum at 21 days after challenge inoculation and it was significantly higher in Pf1 mixed with chitin bioformulation treatment when compared to all other treatments. Increased activity was observed upto 21 days after challenge inoculation and started declined 35 days after challenge inoculation. PGPR untreated green gram plants inoculated with *M. phaseolina* recorded the less induction of PPO throughout the study period and showed a steep decline 21 days after challenge inoculation with *M. phaseolina* (Table 7) (Fig. 2).

4.4.2.3. Phenylalanine ammonia lyase (PAL)

In green gram, *P. fluorescens* Pf1 along with chitin treatment followed by challenge inoculation with *M. phaseolina* induced the plant to synthesize higher levels of PAL. The enzyme activity induced by Pf1 along with chitin bioformulation reached the maximum at 35 days after challenge inoculation and thereafter it decreased. However, PAL activity was significantly higher in PGPR treatment when compared to untreated control (Table 8) (Fig. 3).

4.4.2.4. Chitinase

The activity of chitinase was observed at higher levels in roots of green gram plants pretreated with Pf1 combination with chitin and challenge inoculation with root rot pathogen. The maximum activity was recorded at 21 days after challenge inoculation and it started decline 35 days after challenge inoculation towards the end of experiment.

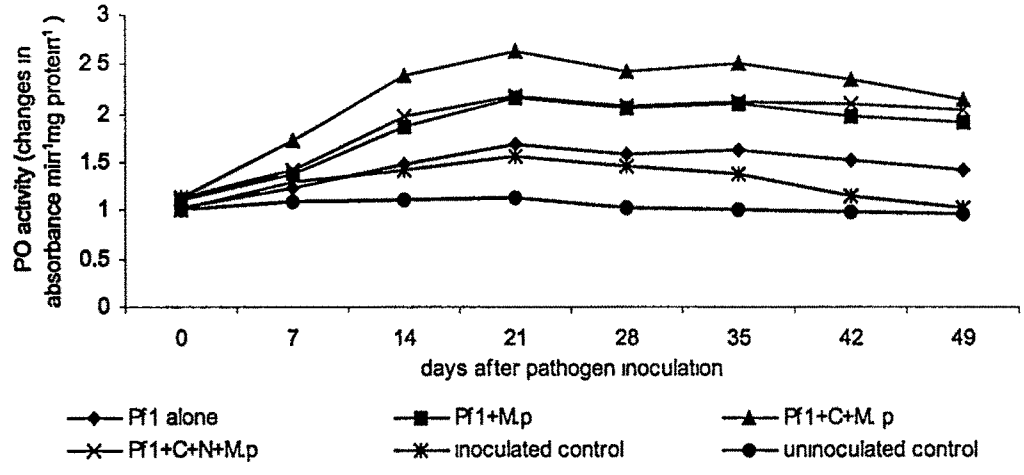
Table 6. Induction of peroxidase activity in green gram roots treated with PGPR strains against root rot disease under greenhouse conditions

Treatments	Days after pathogen inoculation (Changes in absorbance min $^{-1}$ mg^{-1} protein)							
	0	7	14	21	28	35	42	49
Pfl (P)	1.10^{cd}	1.37^{bc}	1.86^{d}	2.15^{b}	2.05^{b}	2.10^{b}	1.98^{c}	1.92^{c}
Pfl + Chitin (C)	1.12^{bc}	1.73^{a}	2.38^{a}	2.63^{a}	2.43^{a}	2.51^{a}	2.34^{a}	2.13^{a}
Pfl + Neem (N)	1.02^{e}	1.32^{cde}	1.65^{f}	2.01^{d}	1.89^{c}	1.96^{c}	1.83^{d}	1.75^{e}
Pfl + C + N	1.14^{bc}	1.42^{b}	1.98^{b}	2.17^{b}	2.07^{b}	2.12^{b}	2.09^{b}	2.03^{b}
Bacillus (B)	1.10^{cd}	1.26^{fg}	1.52^{g}	1.80^{g}	1.76^{e}	1.79^{e}	1.72^{e}	1.67^{f}
B + C	1.25^{a}	1.42^{b}	1.92^{c}	2.07^{c}	1.89^{c}	2.08^{b}	1.99^{c}	1.94^{c}
B + N	1.15^{bc}	1.26^{fg}	1.42^{h}	1.71^{h}	1.65^{f}	1.70^{f}	1.59^{f}	1.55^{g}
B + N + C	1.05^{de}	1.39^{b}	1.71^{e}	1.94^{ef}	1.85^{cd}	1.92^{cd}	1.86^{d}	1.83^{d}
P + B	1.18^{b}	1.30^{fdef}	1.85^{d}	1.95^{e}	1.80^{de}	1.87^{d}	1.83^{d}	1.79^{de}
P + B + C	1.17^{b}	1.42^{b}	1.96^{bc}	2.16^{b}	2.04^{b}	2.08^{b}	1.98^{c}	1.82^{d}
P + B + N	1.13^{bc}	1.23^{g}	1.43^{h}	1.69^{h}	1.61^{fg}	1.67^{f}	1.56^{f}	1.48^{h}
P+ B + N + C	1.09^{cd}	1.36^{bcd}	1.70^{ef}	1 89^{f}	1.83^{d}	1.88^{d}	1.71^{e}	1.62^{f}
Carbendazım	1.02^{e}	1.31^{cdef}	1.43^{h}	1.60^{i}	1.56^{g}	1.58^{g}	1.49^{g}	1.35^{i}
Control	1.00^{e}	1.29^{ef}	1.41^{h}	1.56^{i}	1.46^{h}	1.36^{h}	1.15^{h}	1.02^{j}

Values are mean of two replications

In a column, means followed by a common letter are not significantly different (p=0.05) by DMRT

Fig 1. Induction of peroxidase activity in roots treated with Pf1 bioformulations against root rot of green gram



Pf1 - *P. fluorescens*
M. p. – *Macrophomina phaseolina*
C – Chitin
N – Neem

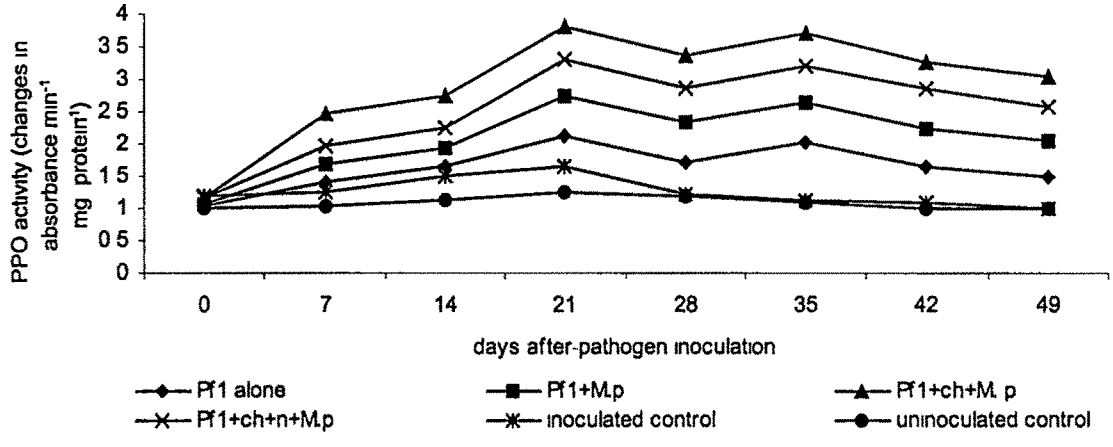
Table 7. Induction of polyphenol oxidase activity in green gram roots treated with PGPR strains against root rot disease under greenhouse conditions

Treatments	Days after pathogen inoculation (Changes in absorbance min $^{-1}$ mg^{-1} protein							
	0	7	14	21	28	35	42	49
Pf1 (P)	1.05^{e}	1.62^{c}	1.91^{e}	2.70^{c}	2.33^{d}	2.65^{d}	2.23^{d}	2.05^{c}
Pf1 + Chitin(C)	1.15^{c}	2.39^{a}	2.66^{a}	3.72^{a}	3.30^{a}	3.62^{a}	3.27^{a}	3.06^{a}
Pf1 + Neem (N)	1.08^{e}	1.59^{cd}	2.15^{d}	2.65^{c}	2.31^{d}	2.45^{f}	2.15^{e}	1.86^{d}
Pf1 + C + N	1.15^{c}	1.97^{b}	2.24^{c}	3.30^{b}	2.88^{b}	3.20^{b}	2.87^{b}	2.58^{b}
Bacillus (B)	1.17^{c}	1.45^{e}	1.89^{e}	2.24^{e}	2.06^{e}	2.38^{g}	2 01^{f}	1.79^{e}
B + C	1.29^{a}	1.60^{cd}	1.90^{e}	2.69^{c}	2.31^{d}	2.58^{e}	2.13^{d}	2.01^{d}
B + N	1.09^{de}	1.35^{f}	1.63^{g}	1.96^{f}	1.79^{f}	1.91^{i}	1.68^{h}	1.45^{f}
B + N + C	1.14^{cd}	1.49^{e}	1.71^{f}	2.68^{c}	2.30^{d}	2.28^{h}	1.99^{e}	1.87^{e}
P + B	1.07^{e}	1.56^{d}	2.19^{c}	2.67^{c}	2.45^{c}	2.59^{e}	2.10^{fg}	1.74^{d}
P + B + C	1.05^{e}	1.96^{b}	2.53^{b}	3.25^{b}	2.83^{b}	3.10^{c}	2.83^{c}	2.43^{b}
P + B + N	1.18^{bc}	1.36^{f}	1.60^{g}	1.94^{f}	1.7^{g}	1.89^{I}	1.62^{i}	1.39^{g}
P+ B + N + C	1.23^{b}	1.39^{f}	1.70^{f}	2.58^{d}	2.48^{c}	2.63^{de}	2.04^{g}	1.72^{e}
Carbendazim	1.14^{cd}	1.29^{g}	1.58^{g}	1.68^{g}	1.59^{h}	1.63^{J}	1.51^{J}	1.10^{h}
Control	1.18^{bc}	1.24^{g}	1.51^{h}	1.65^{g}	1.23^{i}	1.12^{k}	1.08^{k}	0.98^{i}

Values are mean of two replications

In a column, means followed by a common letter are not significantly different (p=0.05) by DMRT

Fig 2. Induction of polyphenol oxidase activity in roots treated with Pf1 bioformulations against root rot of green gram



Pf1 - *P. fluorescens*
M p. – *Macrophomina phaseolina*
C – Chitin
N – Neem

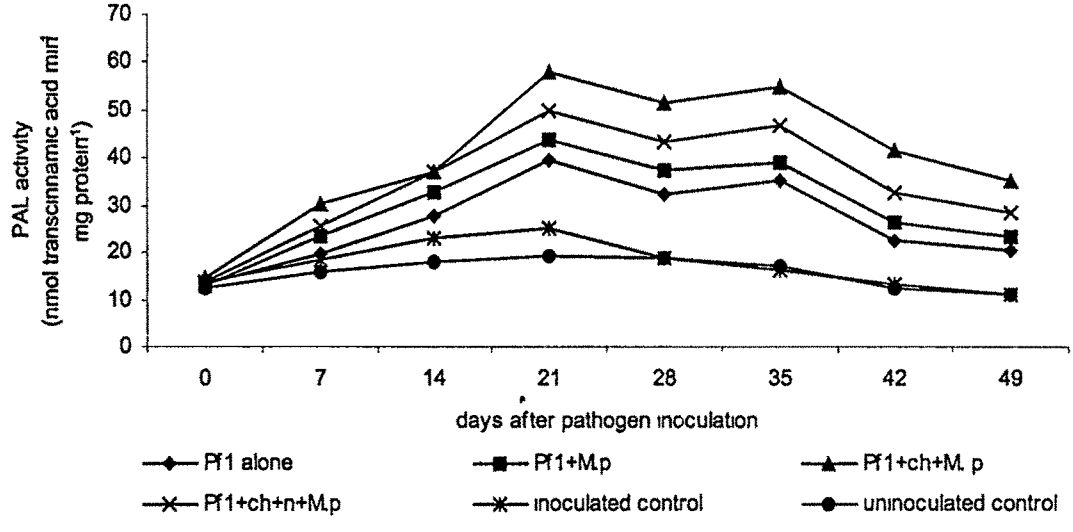
Table 8. Induction of PAL enzyme activity in green gram roots treated with PGPR strains against root rot disease under greenhouse conditions

Treatments	Days after pathogen inoculation (n mol transcinnamic acid min^{-1} mg^{-1} protein)							
	0	7	14	21	28	35	42	49
Pfl (P)	12.85^{h}	23.65^{d}	32.85^{c}	43.86^{d}	37.51^{d}	39.25^{c}	26.54^{d}	23.59^{d}
Pfl + Chitin (C)	14.51^{c}	30.25^{a}	37.14^{a}	58.12^{a}	51.73^{a}	54.91^{a}	41.85^{a}	35.62^{a}
Pfl + Neem (N)	13.25^{g}	20.95^{e}	28.96^{f}	39.62^{g}	34.01^{g}	36.21^{f}	22.42^{g}	19.25^{g}
Pfl + C + N	13.58^{ef}	25.46^{b}	37.02^{a}	49.86^{b}	43.50^{b}	47.02^{b}	32.85^{b}	28.58^{b}
Bacillus (B)	13.69^{e}	19.56^{h}	27.56^{g}	38.25^{h}	32.95^{i}	35.10^{h}	20.97^{i}	18.21^{h}
B + C	14.68^{b}	20.42^{f}	29.36^{e}	40.13^{f}	36.02^{f}	37.25^{e}	23.15^{f}	20.26^{f}
B + N	14.02^{d}	18.96^{i}	26.50^{h}	37.02^{i}	32.56^{j}	33.25^{i}	19.65^{k}	18.02^{i}
B + N + C	14.89^{a}	19.98^{g}	26.25^{i}	39.56^{g}	33.56^{h}	36.25^{f}	22.15^{h}	19.16^{g}
P + B	12.28^{i}	21.03^{e}	31.25^{d}	41.53^{e}	36.56^{e}	38.24^{d}	24.23^{e}	21.32^{e}
P + B + C	13.25^{g}	25.06^{c}	36.25^{b}	48.69^{c}	42.95^{c}	47.03^{b}	31.96^{c}	27.26^{c}
P + B + N	15.02^{a}	18.02^{k}	25.09^{k}	36.25^{j}	31.25^{k}	30.58^{j}	18.25^{l}	16.35^{k}
P+ B + N + C	14.98^{a}	19.02^{i}	25.96^{j}	38.25^{h}	32.85^{i}	35.98^{g}	20.15^{i}	18.25^{h}
Carbendazım	13.69^{e}	18.25^{j}	24.01^{l}	26.03^{k}	23.15^{l}	25.16^{k}	20.15^{j}	16.92^{j}
Control	13.54^{f}	18.25^{j}	23.23^{m}	25.26^{l}	18.90^{m}	16.25^{l}	13.25^{m}	11.24^{l}

Values are mean of two replications

In a column, means followed by a common letter are not significantly different (p=0.05) by DMRT

Fig 3. Induction of PAL activity in roots treated with Pf1 bioformulations against root rot of green gram



Pf1 - *P. fluorescens*
M. p. – *Macrophomina phaseolina*
C – Chitin
N – Neem

However, the activity was significantly higher in Pf1 combination with chitin treatment than the untreated control (Table 9) (Fig. 4).

4.4.2.5. β-1, 3-glucanase

The activity of β-1, 3-glucanase increased in plants treated with bioformulation mixture (Pf1 + chitin) upto 21 days of challenge inoculation with *M. phaseolina*. Later, 35 days of inoculation with pathogen, inoculated control decreased to the lower levels of glucanase as that of uninoculated control (Table 10) (Fig. 5).

4.4.2.6. Phenols

Higher levels of accumulation of phenolics was observed in green gram roots upto 28 days which was treated with the bioformulation mixture of Pf1 along with chitin and challenge inoculated with *M. phaseolina*. In PGPR untreated plants increased phenolic content was noticed upto 21 days after challenge inoculation and thereafter it decreased drastically and remained at lower levels throughout the experimental period (Table 11) (Fig. 6).

4.4.2.7. SDS-PAGE analysis of crude protein of *P. fluorescens* treated plants

The protein-banding pattern was studied from the green gram plants treated with *P. fluorescens* alone and in combination with chitin bioformulation. Banding pattern of protein was predominant in *P. fluorescens* Pf1 in combination with chitin when compared to inoculated control. *P. fluorescens* Pf1 along with chitin, induced proteins with molecular weight of 45 and 65 and several new proteins appeared with high intensity (Plate 6).

4.4.2.8. Isoform pattern of peroxidase (PO)

Native gel electrophoretic separation of enzyme extract from *Pseudomonas* and *Bacillus* treated plants showed different PO isoforms. PGPR strains treated plants, after challenge inoculation with *M. phaseolina* showed 4 isoforms *viz.*, PO1, PO2, PO3 and PO4, but the pathogen alone inoculated control plants revealed only three isoforms and these isoforms were with less intensity. The induction of peroxidase isoforms were more

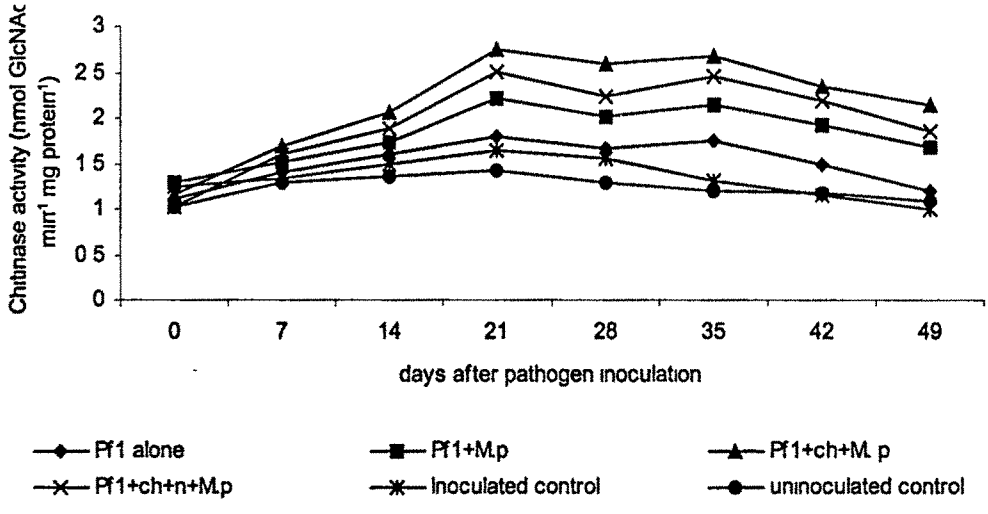
Table 9. Induction of chitinase activity in green gram roots treated with PGPR strains against root rot disease under greenhouse conditions

Treatments	Days after pathogen inoculation (n mol GlcNac $min^{-1}mg^{-1}$ of protein)							
	0	7	14	21	28	35	42	49
Pfl (P)	1.28^{bc}	1.50^{c}	1.72^{c}	2.21^{d}	2.02^{c}	2.15^{d}	1.93^{cd}	1.69^{cd}
Pfl + Chitin (C)	1.15^{d}	1.69^{a}	2.05^{a}	2.75^{a}	2.59^{a}	2.69^{a}	2.35^{a}	2.15^{a}
Pfl + Neem (N)	1.15^{b}	1.43^{d}	1.65^{de}	2.01^{f}	1.89^{e}	1.99^{f}	1.90^{d}	1.52^{f}
Pfl + C + N	1.02^{e}	1.60^{b}	1.89^{b}	2.51^{b}	2.23^{b}	2.46^{b}	2.19^{b}	1.85^{b}
Bacillus (B)	1.16^{d}	1.35^{ef}	1.60^{ef}	1.99^{fg}	1.91^{de}	1.95^{f}	1.76^{e}	1.52^{f}
B + C	1.24^{c}	1.45^{cd}	1.72^{c}	2.08^{e}	2.03^{c}	2.05^{e}	1.81^{e}	1.71^{c}
B + N	1.12^{d}	1.30^{f}	1.55^{fg}	1.94^{g}	1.84^{fg}	1.86^{g}	1.70^{f}	1.47^{f}
B + N + C	1.28^{bc}	1.41^{d}	1.69^{cd}	2.02^{f}	1.95^{d}	1.99^{f}	1.78^{e}	1.65^{de}
P + B	1.26^{bc}	1.39^{de}	1.65^{de}	2.04^{ef}	1.87^{ef}	1.97^{f}	1.82^{e}	1.65^{de}
P + B + C	1.23^{c}	1.59^{b}	1.86^{b}	2.49^{b}	2.20^{b}	2.41^{b}	2.16^{b}	1.80^{b}
P + B + N	1.25^{bc}	1.30^{f}	1.59^{f}	1.95^{g}	1.79^{g}	1.86^{g}	1.80^{e}	1.61^{e}
P+ B + N + C	1.34^{a}	1.42^{d}	1.73^{c}	2.29^{c}	2.01^{c}	2.21^{c}	1.96^{c}	1.85^{b}
Carbendazim	1.31^{ab}	1.32^{f}	1.51^{gh}	1.71^{h}	1.65^{h}	1.69^{h}	1.52^{g}	1.39^{g}
Control	1.25^{bc}	1.32^{f}	1.48^{h}	1.64^{i}	1.56^{i}	1.31^{i}	1.15^{h}	1.01^{h}

Values are mean of two replications

In a column, means followed by a common letter are not significantly different (p=0.05) by DMRT

Fig 4. Induction of chitinase activity in roots treated with Pf1 bioformulations against root rot of green gram



Pf1 - *P. fluorescens*
M. p. – *Macrophomina phaseolina*
C – Chitin
N – Neem

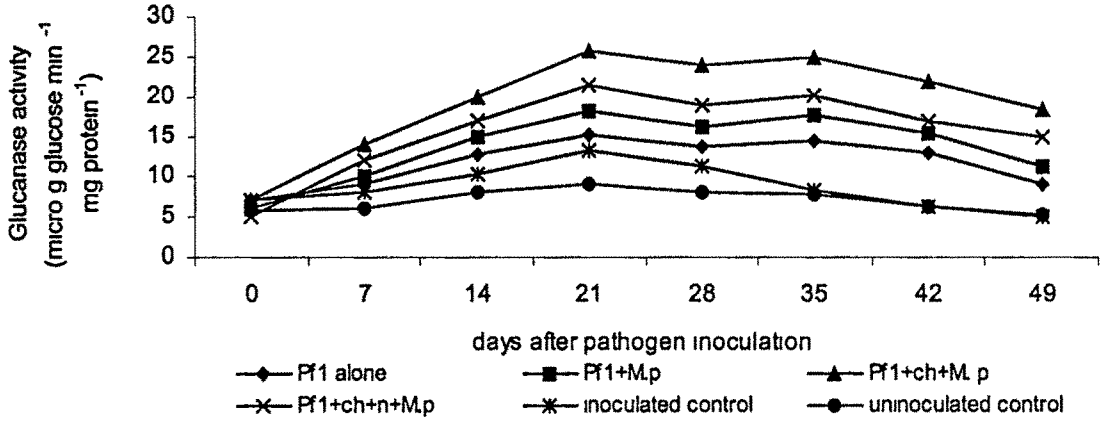
Table 10. Induction of β-1,3-glucanase activity in green gram roots treated with PGPR strains against root rot disease under greenhouse conditions

Treatments	Days after pathogen inoculation (μg of glucose min^{-1} mg^{-1} protein)							
	0	7	14	21	28	35	42	49
Pfl (P)	6.01^{e}	10.01^{e}	15.02^{d}	18.35^{e}	16.25^{e}	17.85^{e}	15.56^{e}	11.25^{e}
Pfl + Chitin (C)	7.12^{a}	13.95^{a}	20.03^{a}	25.63^{a}	23.95^{a}	24.90^{a}	21.95^{a}	18.42^{a}
Pfl + Neem (N)	6.95^{b}	9.02^{h}	13.25^{h}	16.58^{i}	15.02^{g}	16.03^{h}	13.52^{h}	10.04^{h}
Pfl + C + N	4.89^{j}	11.99^{b}	16.99^{b}	21.45^{b}	19.01^{b}	20.14^{b}	17.02^{b}	15.02^{b}
Bacillus (B)	6.94^{b}	8.89^{i}	11.26^{i}	14.26^{k}	13.98^{l}	14.17^{k}	10.25^{k}	9.23^{j}
B + C	4.95^{ij}	10.23^{f}	14.05^{f}	17.26^{g}	16.23^{e}	17.24^{f}	14.23^{g}	11.24^{e}
B + N	5.54^{g}	8.02^{k}	10.23^{k}	13.24^{l}	11.02^{l}	12.58^{m}	9.63^{l}	8.96^{k}
B + N + C	5.86^{f}	9.35^{g}	13.45^{g}	16.24^{j}	14.96^{g}	15.92^{i}	13.24^{i}	10.82^{f}
P + B	6.21^{d}	9.59^{e}	14.23^{e}	17.35^{f}	15.26^{f}	16.85^{g}	15.02^{f}	10.34^{g}
P + B + C	5.26^{h}	10.99^{c}	15.26^{c}	20.42^{c}	18.27^{c}	19.96^{c}	16.85^{c}	14.52^{c}
P + B + N	6.51^{c}	9.02^{f}	14.09^{f}	16.92^{h}	14.27^{h}	15.34^{j}	13.25^{i}	9.31^{l}
P+ B + N + C	4.99^{i}	10.96^{c}	15.27^{c}	19.63^{d}	17.26^{d}	18.69^{d}	15.75^{d}	13.72^{d}
Carbendazim	6.26^{d}	7.56^{j}	10.94^{j}	14.21^{k}	12.03^{j}	13.25^{l}	10.23^{j}	5.26^{l}
Control	6.96^{b}	7.96^{k}	10.27^{k}	13.24^{l}	11.26^{k}	8.25^{n}	6.31^{m}	4.96^{m}

Values are mean of two replications

In a column, means followed by a common letter are not significantly different (p=0.05) by DMRT

Fig 5. Induction of β-1, 3-glucanase activity in roots treated with Pf1 bioformulations against root rot of green gram



Pf1 - *P. fluorescens*
M. p. – *Macrophomina phaseolina*
C – Chitin
N – Neem

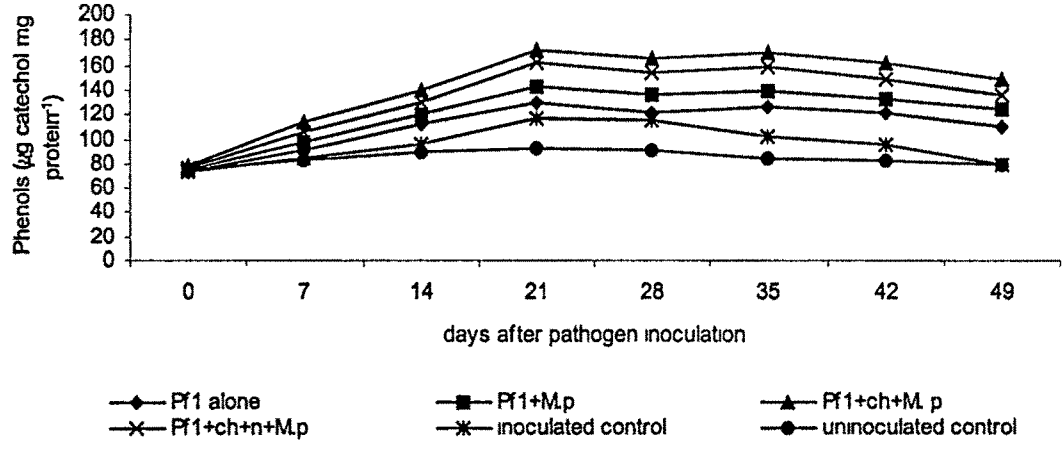
Table 11. Induction of phenols in green gram roots treated with PGPR strains against root rot disease under greenhouse conditions

Treatments	Days after pathogen inoculation (µg of catechol mg $^{-1}$ of protein)							
	0	7	14	21	28	35	42	49
Pfl (P)	74.56^{abc}	98.25^{c}	119.65^{c}	136.23^{c}	142.36^{c}	140.25^{c}	132.56^{c}	124.53^{d}
Pfl + Chitin (C)	77.26^{a}	114.26^{a}	139.85^{a}	165.38^{a}	172.56^{a}	170.25^{a}	162.58^{a}	149.98^{a}
Pfl + Neem (N)	70.23^{d}	92.52^{d}	105.26^{de}	123.02^{de}	132.56^{de}	126.3^{ef}	120.36^{e}	116.35^{f}
Pfl + C + N	75.96^{ab}	105.85^{b}	130.25^{b}	154.26^{b}	163.21^{b}	159.87^{b}	149.25^{b}	135.98^{b}
Bacillus (B)	76.35^{ab}	90.36^{de}	103.25^{e}	123.69^{d}	130.25^{ef}	124.98^{efg}	118.75^{ef}	114.26^{fg}
B + C	74.26^{abc}	93.26^{d}	107.02^{d}	125.09^{d}	134.82^{d}	130.85^{d}	124.36^{d}	120.35^{e}
B + N	74.82^{abc}	87.96^{ef}	105.83^{de}	120.53^{e}	127.56^{f}	122.85^{g}	114.25^{gh}	111.02^{h}
B + N + C	76.42^{ab}	90.25^{de}	105.26^{de}	123.98^{d}	132.89^{de}	127.78^{e}	120.73^{e}	116.91^{f}
P + B	75.26^{abc}	97.82^{c}	119.26^{c}	135.75^{c}	140.25^{c}	139.86^{c}	130.75^{c}	120.37^{e}
P + B + C	74.91^{abc}	104.26^{b}	129.56^{b}	154.03^{b}	160.42^{b}	157.36^{b}	147.25^{b}	132.05^{c}
P + B + N	76.08^{ab}	87.59^{ef}	104.25^{de}	116.35^{f}	124.36^{g}	117.25^{h}	111.27^{i}	103.46^{i}
P+ B + N + C	75.63^{abc}	89.36^{e}	104.23^{de}	120.56^{e}	130.26^{ef}	124.25^{fg}	116.58^{gh}	112.82^{gh}
Carbendazim	72.52^{cd}	86.23^{f}	98.36^{f}	117.23^{f}	120.25^{h}	119.36^{h}	113.25^{j}	90.56^{j}
Control	73.65^{bc}	85.23^{f}	96.35^{f}	115.26^{f}	117.25^{i}	102.36^{i}	95.63^{k}	80.23^{k}

Values are mean of two replications

In a column, means followed by a common letter are not significantly different (p=0.05) by DMRT

Fig 6. Accumulation of phenols in roots treated with Pf1 bioformulations against root rot of green gram



Pf1 - *P. fluorescens*
M p. – *Macrophomina phaseolina*
C – Chitin
N – Neem

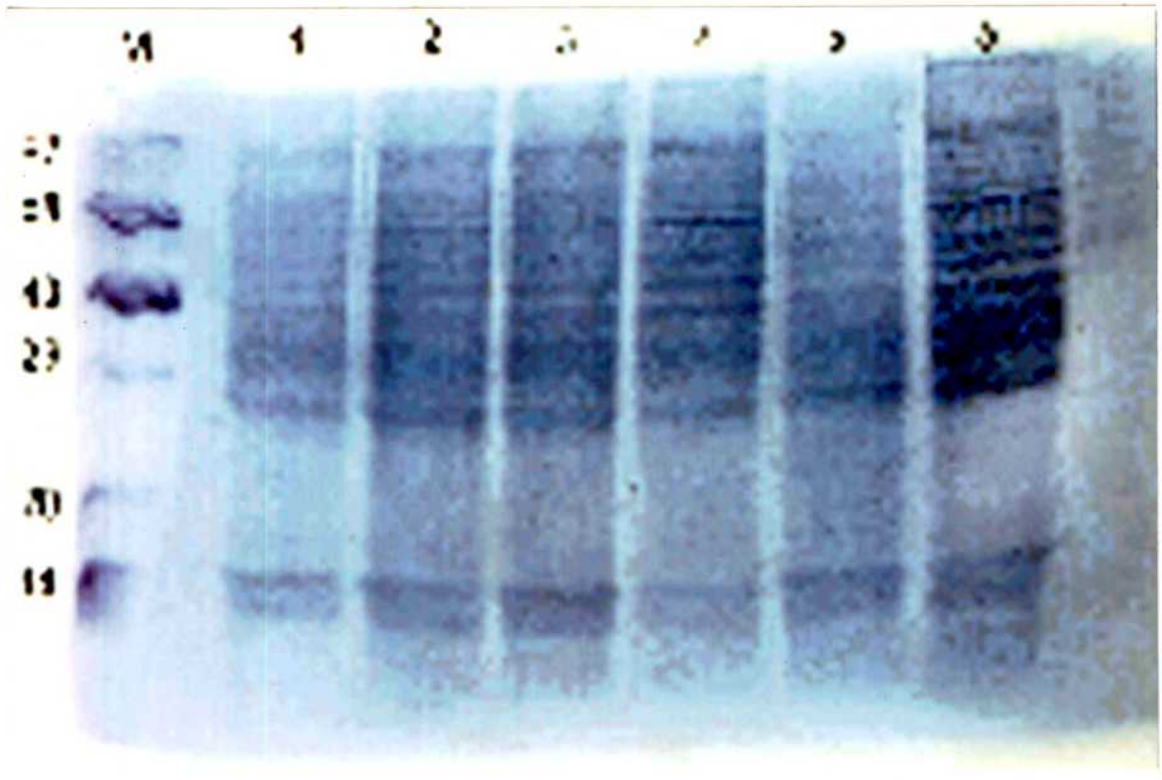
Plate 6. SDS-PAGE analysis of crude protein extract of roots of green gram plants treated with *P. fluorescens* bioformulations challenge inoculated with *M. phaseolina*

Lane

M – Marker

1. Untreated control
2. Pf1
3. Pf1 + Neem
4. Pf1 + Neem + Chitin
5. Healthy
6. Pf1 + Chitin

Plate 6



prominent in the treatment involving bioformulations with chitin than without chitin (Plate 7, 8).

4.4.2.9. Isoform pattern of polyphenol oxidase (PPO)

PGPR strains treated plants showed different PPO patterns. Three isoforms *viz.*, PPO1, PPO2 and PPO3 were observed in PGPR treated plants after inoculation with *M. phaseolina* where as in control only two isoforms were noticed and these isoforms were with less intensity. Higher induction of PPO was observed in plants pretreated with Pf1 along with chitin and challenge inoculated with *M. phaseolina*. Lesser induction of PPO isoforms was noticed in the treatments not amended with chitin (Plate 9, 10).

4.4.2.10. Isoform pattern of chitinase

Western blot detected two new chitinase isoforms with molecular weight of 45 (chi 1) and 40 (chi) kDa with barley chitinase antiserum. In control plants inoculated with *M. phaseolina* only two chitinases were detected and they were also not well pronounced in intensity as compared to the Pf1 along with chitin treated plants challenged with the pathogen (Plate 11).

4.4.3. Field experiments

4.4.3.1. Efficacy of bioformulations on dry root rot of green gram

Results of field experiments revealed that the minimum root rot incidence of 1.3, 2.6 and 9.3 per cent was recorded in the trial plots applied with the bioformulation of Pf1 amended with chitin at 30, 45 and 60 days after sowing respectively. Though Pf1 alone significantly reduced the disease incidence when compared to untreated control, it's amendment with chitin showed highest per cent reduction in disease control followed by Pf1 amended with chitin and neem (Table 12) (Plate 12).

4.4.3.2. Efficacy of PGPR strains on yield of green gram

Yield attributing parameters *viz.*, number of seeds per pod, number of pods per plant were recorded. Significantly higher number of seeds per pod and number of pods per plant were recorded from the trial plots treated with Pf1 along with chitin followed by

Plate 7. Native gel electrophoresis of peroxidase induction in roots treated with *P. fluorescens* along with chitin bioformulation mixture against root rot of green gram

Lane

1. Pf1
2. Pf1 + Neem
3. Pf1 + Neem + Chitin
4. Healthy
5. Pf1 + Chitin
6. Control

Plate 7

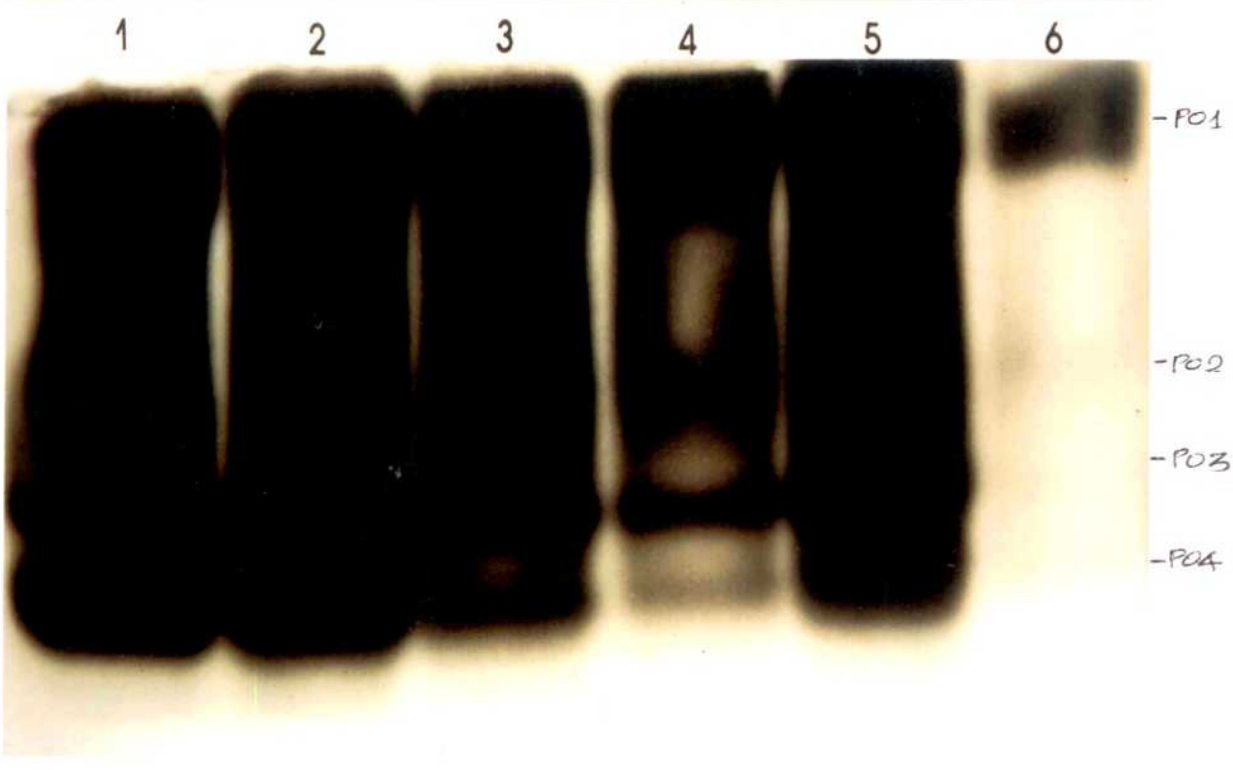


Plate 8. Native gel electrophoresis of peroxidase induction in roots treated with different PGPR bioformulations against root rot of green gram

Lane

1. Pf1 alone
2. Pf1 + *M. phaseolina*
3. Pf1 + Chitin + *M. phaseolina*
4. Pf1 + Neem + *M. phaseolina*
5. Pf1 + Chitin + Neem + *M. phaseolina*
6. B + Chitin + *M. phaseolina*
7. B + Neem + *M. phaseolina*
8. B + Chitin + Neem + *M. phaseolina*
9. Pf1 + B + Chitin + *M. phaseolina*
10. Pf1 + B + Neem + *M. phaseolina*
11. Pf1 + B + Chitin + Neem + *M. phaseolina*
12. Healthy
13. Control

Plate 8

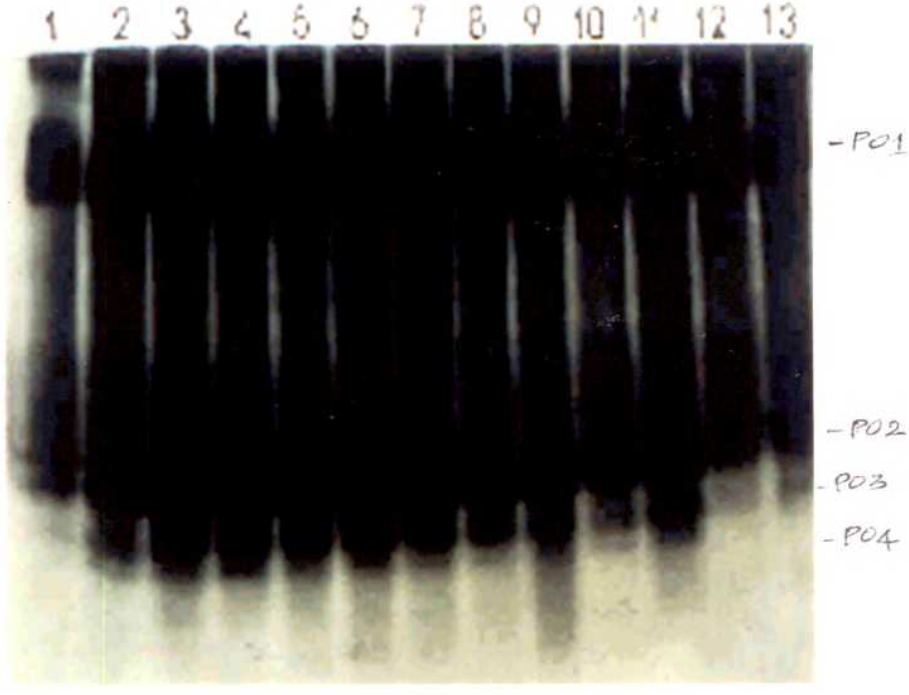


Plate 9. Native gel electrophoresis of polyphenol oxidase induction in roots treated with *P. fluorescens* along with chitin bioformulation mixture against root rot of green gram

Lane

1. Pf1
2. Pf1 + Neem
3. Pf1 + Chitin + Neem
4. Healthy
5. Pf1 + Chitin
6. Control

Plate 9

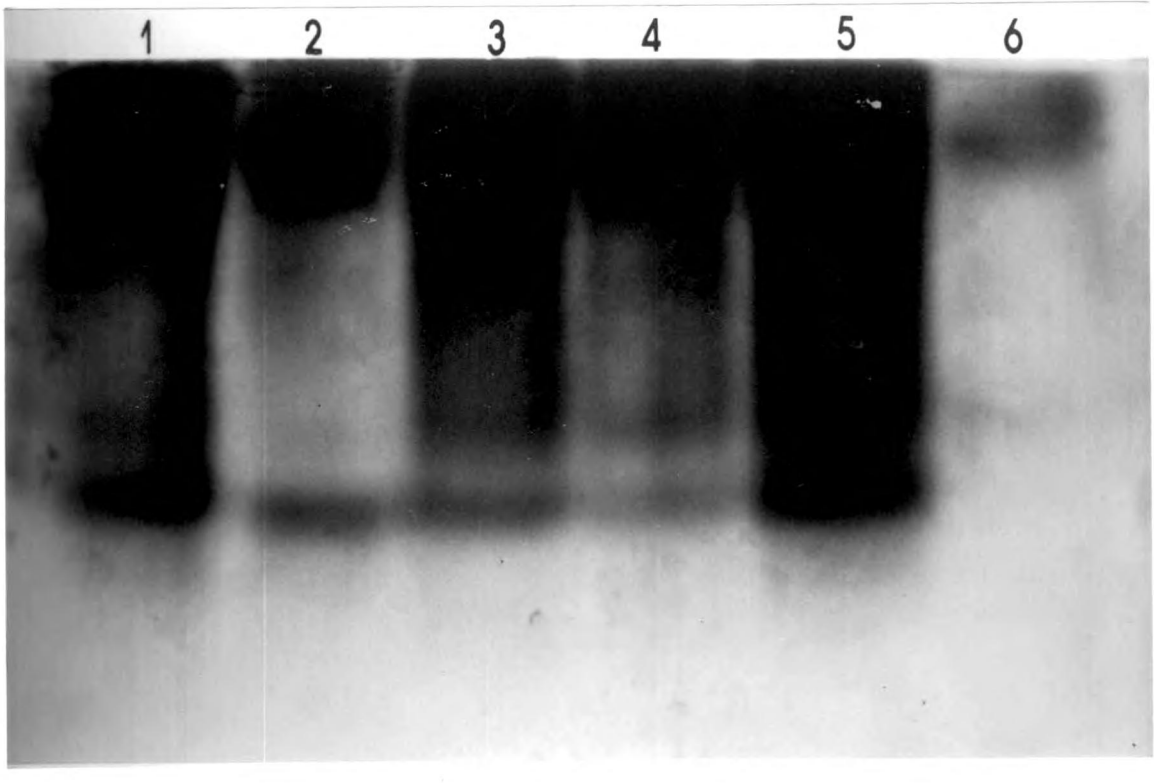


Plate 10. Native gel electrophoresis of polyphenol oxidase induction in roots treated with different PGPR bioformulations against root rot of green gram

Lane

1. Pf1 alone
2. Pf1 + *M. phaseolina*
3. Pf1 + Chitin + *M. phaseolina*
4. Pf1 + Neem + *M. phaseolina*
5. Pf1 + Chitin + Neem + *M. phaseolina*
6. B + Chitin + *M. phaseolina*
7. B + Neem + *M. phaseolina*
8. B + Chitin + Neem + *M. phaseolina*
9. Pf1 + B + Chitin + *M. phaseolina*
10. Pf1 + B + Neem + *M. phaseolina*
11. Pf1 + B + Chitin + Neem + *M. phaseolina*
12. Healthy
13. Control

Plate 10

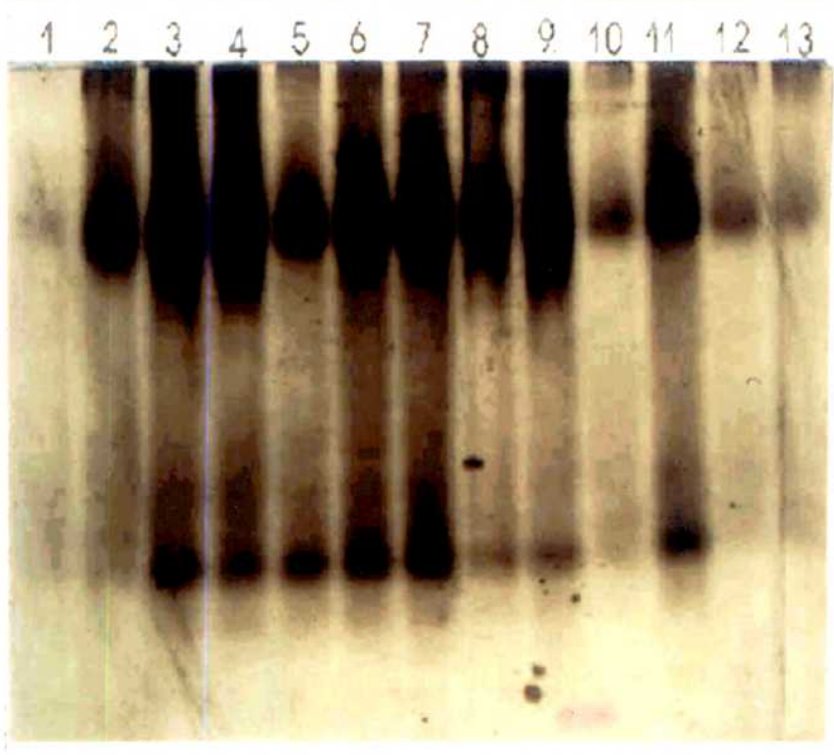


Plate 11. Western blot analysis of crude protein extract probed with barley chitinase antiserum of green gram roots inoculated with *M. phaseolina*

Lane

1. Control
2. Pf1 + *M. phaseolina*
3. Pf1 + Chitin + *M. phaseolina*

Plate 11

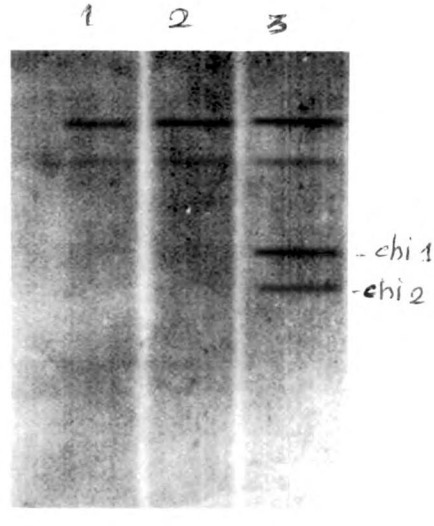


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Table 12. Efficacy of *P. fluorescens* (Pf1) on dry root rot of green gram under field conditions

Treatments	30 DAS	45 DAS	60 DAS	Mean	Per cent reduction over control
P. fluorescens Pf1	2.6 (9.27)^{c}	5.3 (13.30)^{c}	16.0 (23.57)^{c}	7.96^{d}	67.65
Pf 1 + Chitin	1.3 (6.53)^{d}	2.6 (9.27)^{e}	9.3 (17.75)^{f}	4.40^{f}	82.13
Pf 1 + Neem	4.0 (11.53)^{b}	6.6 (14.88)^{b}	18.6 (25.54)^{b}	9.73^{b}	60.48
Pf1 + Chitin + Neem	2.6 (9.27)^{c}	5.3 (13.30)^{c}	14.6 (22.45)^{d}	7.50^{c}	69.55
Carbendazim	1.3 (6.53)^{d}	4.0 (11.53)^{d}	13.3 (21.38)^{e}	6.20^{e}	74.83
Control	11.3 (19.64)^{a}	20.0 (26.56)^{a}	42.6 (40.74)^{a}	24.63^{a}	0.00

Values are mean of three replications

Values in parentheses are arcsine transformed

In a column, means followed by the same letter are not differ significantly (p=0.05) by DMRT

Plate 12. Efficacy of *P. fluorescens* amended with chitin bioformulations on incidence of dry root of green gram under field conditions

Plate 12

Pf1 in combination with chitin and neem bioformulation. The parameters were positively correlated with the grain yield. Pf1 mixed with chitin treated plots recorded the highest yield of 1218 kg/ha which is significantly different from all other treatments including chemical treatment (800 kg/ha). Lowest yield was recorded in the untreated control plots (583 kg/ha). In general all the treatments were effective in increasing the grain yield of green gram (Table 13).

Pf1 combination with chitin maintained its superiority in increasing the yield and in reducing the disease incidence.

4.4.3.3. Assessment of *P. fluorescens* colonization in rhizosphere of green gram

The survival of *P. fluorescens* strain Pf1 was estimated from the rhizosphere of green gram at 30, 45 and 60 DAS. Maximum population of Pf1 (colony forming unit) was recorded in the treatment Pf1 along with chitin. Pf1 along with chitin and neem was observed to the next best treatment for the survival and colonization of Pf1. The lowest population was recorded in untreated control (Table 14).

4.5. Efficacy of bioformulations against blister blight of tea

4.5.1. PGPR induced defense related protein and chemicals against the fungal pathogen *Exobasidium vexans*

4.5.1.1. Peroxidase (PO)

Results revealed that the induction of peroxidase enzymes was significantly higher in tea plants treated with *P. fluorescens* Pf1 at 7 days interval when compared to the untreated control. Two fold increase in peroxidase accumulation was noticed in Pf1 at 7 days interval sprayed plants when compared to untreated control. The next best treatment observed with high induction of PO was *B subtilis* and combination of *P. fluorescens* and *B. subtilis* along with chitin and neem at 7 days interval (Table 15).

4.5.1.2. Polyphenol oxidase (PPO)

Higher induction of polyphenol oxidase was observed in tea plants treated with *P. fluorescens* Pf1 at 7 days interval followed by *B. subtilis* and combination of

Table 13. Efficacy of *P. fluorescens* (Pf1) on yield of green gram under field conditions

Treatment	Number of seeds / pod	Number of pods / plant	Grain yield (kg/ha)	Per cent increase over control
Pf1	10.33[c]	15.33[d]	889	52.48
Pf1 + Chitin (C)	14.66[e]	19.66[f]	1218	108.91
Pf1 + Neem (N)	9.33[b]	14.33[c]	857	46.90
Pf1 + C + N	12.33[d]	17.33[e]	984	68.78
Carbendazim	9.33[b]	13.66[b]	800	37.22
Control	8.66[a]	9.33[a]	583	-

Values are mean of three replications

In a column, means followed by the same letter are not differ significantly (p=0.05) by DMRT

Table 14. Rhizosphere colonization of *P. fluorescens* (Pf1) on green gram roots under field conditions

Treatments	Pf1 population / g of soil (x 10^5)		
	30 DAS	45 DAS	60 DAS
Pf 1	7.14^{c}	8.91^{c}	8.01^{c}
Pf1 + Chitin (C)	8.54^{a}	9.85^{a}	9.20^{a}
Pf1 + Neem (N)	5.91^{d}	6.24^{d}	5.92^{d}
Pf1 + C + N	7.24^{b}	9.01^{b}	8.62^{b}
Carbendazim	4.02^{e}	5.91^{e}	5.51^{e}
Control	1.96^{f}	3.83^{f}	3.61^{f}

Values are mean of three replications

In a column, means followed by the same letter are not differ significantly (p=0.05) by DMRT

Table 15. Induction of peroxidase by PGPR strains against blister blight of tea

Treatments	Changes in absorbance min^{-1} mg^{-1} protein	Per cent increase over control
Pf1 at 7 days interval	1.46^{f}	82.50
Pf1 at 14 days interval	1.21^{d}	51.25
Pf1 at 21 days interval	0.93^{b}	16.25
B. s at 7 days interval	1.28^{de}	60.00
B. s at 14 days interval	1.06^{c}	32.50
B. s at 21 days interval	0.85^{ab}	6.25
Pf1 + *B. s* +C+N at 7 days interval	1.31^{e}	63.75
Pf1 + *B. s* +C+N at 14 days interval	1.12^{c}	40.00
Pf1 + *B. s* +C+N at 21 days interval	0.89^{b}	11.25
Hexaconazole	1.08^{c}	35.00
Control	0.80^{a}	0.00

C- chitin

N- neem

Values are mean of three replications

In a column, means followed by a common letter are not significantly different (p = 0.05) by DMRT

P. fluorescens and *B. subtilis* amended with chitin and neem at 7 days interval. Untreated control revealed the less induction of polyphenol oxidase and it was two fold less compar d to the best treatment (Table 16).

4.5.1.3. Phenylalanine ammonia lyase (PAL)

PAL accumulation was higher in tea plants treated with *P. fluorescens* at 7 days interval and showed the 79.85 per cent increase over the untreated control. The next best treatment observed with higher accumulation of PAL was *B. subtilis* at 7 days interval spray (Table 17).

4.5.1.4. Chitinase

Induction of chitinase enzyme was significantly less in case of untreated control when compared to the tea plants treated with Pf1 at 7 days interval, which showed the higher induction of chitinase activity followed by application of *B. subtilis* and *B subtilis* in combination with *P. fluorescens* Pf1 amended with chitin and neem at 7 days interval. The enzyme activity of 55.62 per cent was increased in tea plants treated with *P. fluorescens* Pf1 sprayed at 7 days interval when compared to untreated control (Table 18).

4.5.1.5. β-1, 3-glucanase

P. fluorescens Pf1 sprayed at 7 days interval resulted in higher induction of glucanase activity in tea plants when compared to the untreated control. Increase in glucanase activity (76.79 per cent) was observed in Pf1 sprayed at 7 days interval tea plants when compared to the untreated control. In general 7 days interval spray of *P fluorescens* and *B. subtilis* either alone or combination with chitin and neem showed the significant increase in enzyme activity than the untreated control (Table 19).

4.5.1.6. Phenolics

Accumulation of phenols in tea plants sprayed with *P fluorescens* Pf1 bioformulation at 7 days interval significantly higher when compared to all other treatment. Highest accumulation of phenols was noticed in tea plants sprayed with

Table 16. Induction of polyphenol oxidase by PGPR strains against blister blight of tea

Treatments	Changes in absorbance min^{-1} mg^{-1} protein	Per cent increase over control
Pf1 at 7 days interval	2.28^{i}	68.88
Pf1 at 14 days interval	1.82^{f}	34.81
Pf1 at 21 days interval	1.55^{c}	14.81
B. s at 7 days interval	2.10^{h}	55.55
B. s at 14 days interval	1.68^{d}	24.44
B. s at 21 days interval	1.38^{ab}	2.22
Pf1 + *B. s* +C+N at 7 days interval	2.13^{h}	57.77
Pf1 + *B. s* +C+N at 14 days interval	1.74^{e}	28.88
Pf1 + *B. s* +C+N at 21 days interval	1.42^{b}	5.18
Hexaconazole	1.90^{g}	40.74
Control	1.35^{a}	0.00

C- chitin

N- neem

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 17. Induction of phenylalanine ammonia lyase by PGPR strains against blister blight of tea

Treatments	n mol transcinnamic acid min^{-1} mg^{-1} protein	Per cent increase over control
Pf1 at 7 days interval	12.32[i]	79.85
Pf1 at 14 days interval	10.23[f]	49.34
Pf1 at 21 days interval	7.83[c]	14.30
B s at 7 days interval	10.61[h]	54.89
B. s at 14 days interval	9.04[e]	31.97
B. s at 21 days interval	7.01[b]	2.33
Pf1 + *B. s* +C+N at 7 days interval	10.48[g]	52.99
Pf1 + *B. s* +C+N at 14 days interval	9.02[e]	31 67
Pf1 + *B s* +C+N at 21 days interval	6.94[b]	1.31
Hexaconazole	8.46[d]	23.50
Control	6.85[a]	0.00

C- chitin

N- neem

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 18. Induction of chitinase by PGPR strains against blister blight of tea

Treatments	n mol GlcNac min^{-1} mg^{-1} of protein	Per cent increase over control
Pf1 at 7 days interval	2.35^{g}	55.62
Pf1 at 14 days interval	1.96^{e}	29.80
Pf1 at 21 days interval	1.78^{cd}	17.88
B. s at 7 days interval	2.18^{f}	44.37
B. s at 14 days interval	1.85^{d}	22.51
B s at 21 days interval	1.63^{b}	7.94
Pf1 + *B. s* +C+N at 7 days interval	2.23^{f}	47.68
Pf1 + *B. s* +C+N at 14 days interval	1.84^{d}	21.85
Pf1 + *B. s* +C+N at 21 days interval	1.61^{b}	6.62
Hexaconazole	1.72^{c}	13.90
Control	1.51^{a}	0.00

C- chitin

N- neem

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 19. Induction of β-1, 3-glucanase by PGPR strains against blister blight of tea

Treatments	µg of glucose min $^{-1}$ mg^{-1} protein	Per cent increase over control
Pf1 at 7 days interval	35.50^{h}	76.79
Pf1 at 14 days interval	29.35^{e}	46.16
Pf1 at 21 days interval	24.20^{c}	20.51
B. s at 7 days interval	30.25^{f}	50.64
B. s at 14 days interval	23.47^{c}	16.88
B. s at 21 days interval	21.59^{b}	7.51
Pf1 + *B. s* +C+N at 7 days interval	31.48^{g}	56.77
Pf1 + *B. s* +C+N at 14 days interval	26.74^{d}	33.16
Pf1 + *B. s* +C+N at 21 days interval	20.82^{ab}	3.68
Hexaconazole	29.00^{e}	44.42
Control	20.08^{a}	0 00

C- chitin

N- neem

Values are mean of three replications

In a column, means followed by a common letter are not significantly different (p = 0.05) by DMRT

bioformulation of *P. fluorescens* Pf1 at 7 days interval when compared to the untreated control. Tea plants treated with *P. fluorescens* Pf1 and *B. subtilis* combination amended with chitin and neem bioformulation at weekly interval induced the phenol accumulation next to tea plants treated *P. fluorescens* Pf1 at 7 days interval (Table 20).

In general, tea plants treated with *P. fluorescens* at weekly intervals showed the higher induction of defense related enzymes against the blister blight disease followed by weekly spray of *B. subtilis* and combination of *P. fluorescens* and *B. subtilis* (Fig. 7, 8).

4.5.2. Efficacy of bioformulations against blister blight disease in tea plantations

A field trial was conducted at Anamallais Hills (Plate 13). Among the various combinations of bioformulation used at different days interval, *P. fluorescens* at weekly interval spray recorded with less mean disease index of 16.92 which was significantly different from untreated control recording the mean disease index of 46.72 during 2001 (Plate 14a, 14b). Results revealed that weekly spray of *P. fluorescens* reduced the disease incidence through out the study period and it was comparable with the chemical fungicide (Fig. 9). In general weekly spray of *P. fluorescens* Pf1 and *B subtilis* alone or in combination with chitin and neem showed the significant reduction in disease index when compared to untreated control (Table 21) (Plate 15a, 15b, 16a, 16b).

During 2002, before and after pruning of tea plants, generally less disease incidence was noticed in trial plots treated with *P. fluorescens* at weekly interval followed by weekly interval spray of *P. fluorescens* and *B. subtilis* combination with chitin and neem when compared to untreated control. *P. fluorescens* Pf1 spray at weekly intervals was comparable with chemical spray in reducing the blister blight of tea plantations (Table 22) (Plate 17a, 17b).

4.5.3. Efficacy of PGPR bioformulations on yield of tea plantations

Results indicated that the *P. fluorescens* at weekly interval spray significantly increased the yield of tea throughout the study period when compared to untreated control and chemical treatment during 2001 (Fig. 10). Similar trend was observed during

Table 20. Accumulation of phenolics by PGPR strains against blister blight of tea

Treatments	µg of catechol mg $^{-1}$ of protein	Per cent increase over control
Pf1 at 7 days interval	113.90^{g}	122.02
Pf1 at 14 days interval	91.75^{e}	78.84
Pf1 at 21 days interval	78.40^{c}	52.82
B. s at 7 days interval	98.55^{f}	92.10
B. s at 14 days interval	87.30^{d}	70.17
B. s at 21 days interval	72.15^{b}	40.64
Pf1 + *B. s* +C +N at 7 days interval	101.35^{f}	97.56
Pf1 + *B. s* +C+N at 14 days interval	88.40^{d}	72.31
Pf1 + *B. s* +C +N at 21 days interval	72.50^{b}	41.32
Hexaconazole	93.40^{e}	82.06
Control	51.30^{a}	0.00

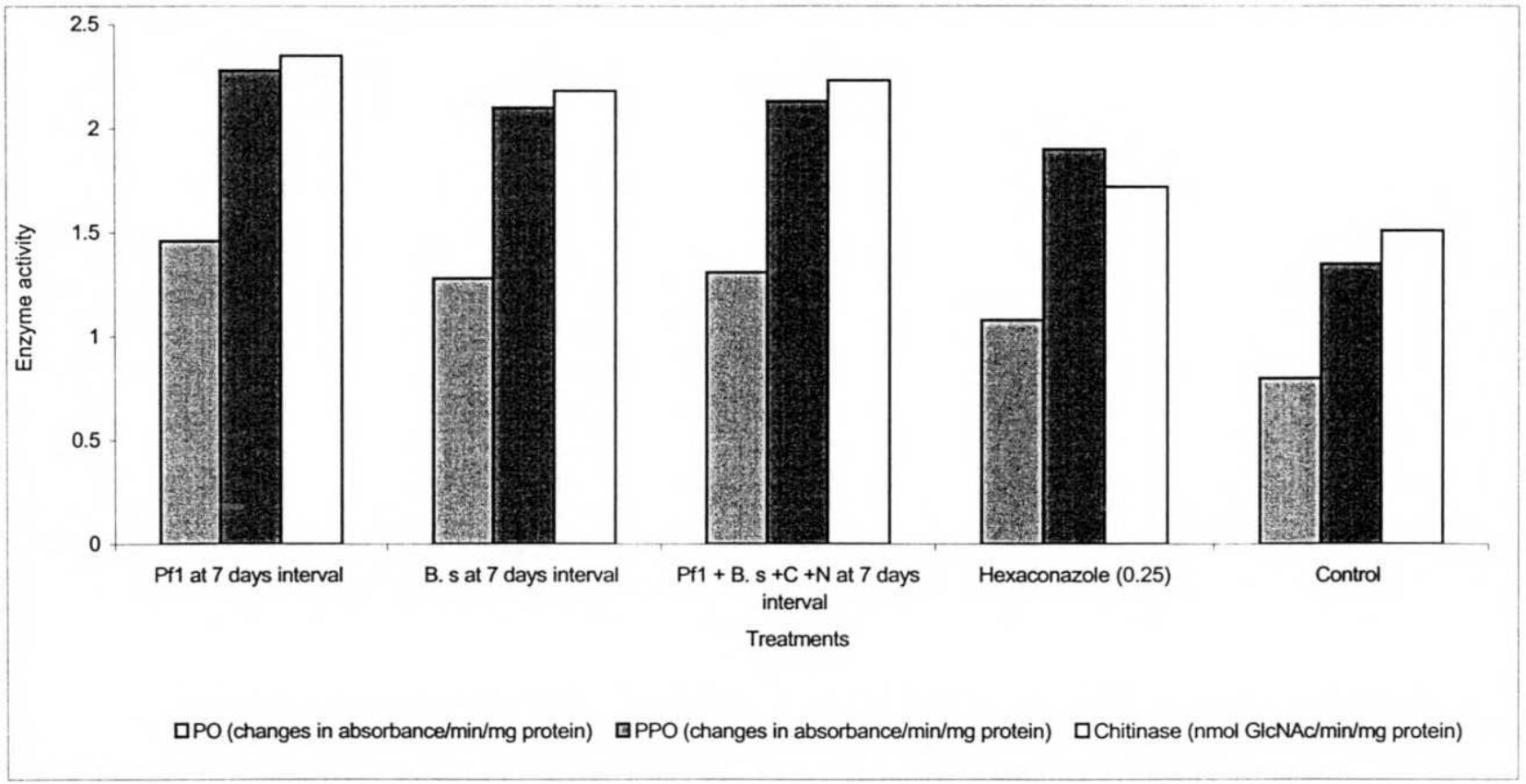
C- chitin

N- neem

Values are mean of three replications

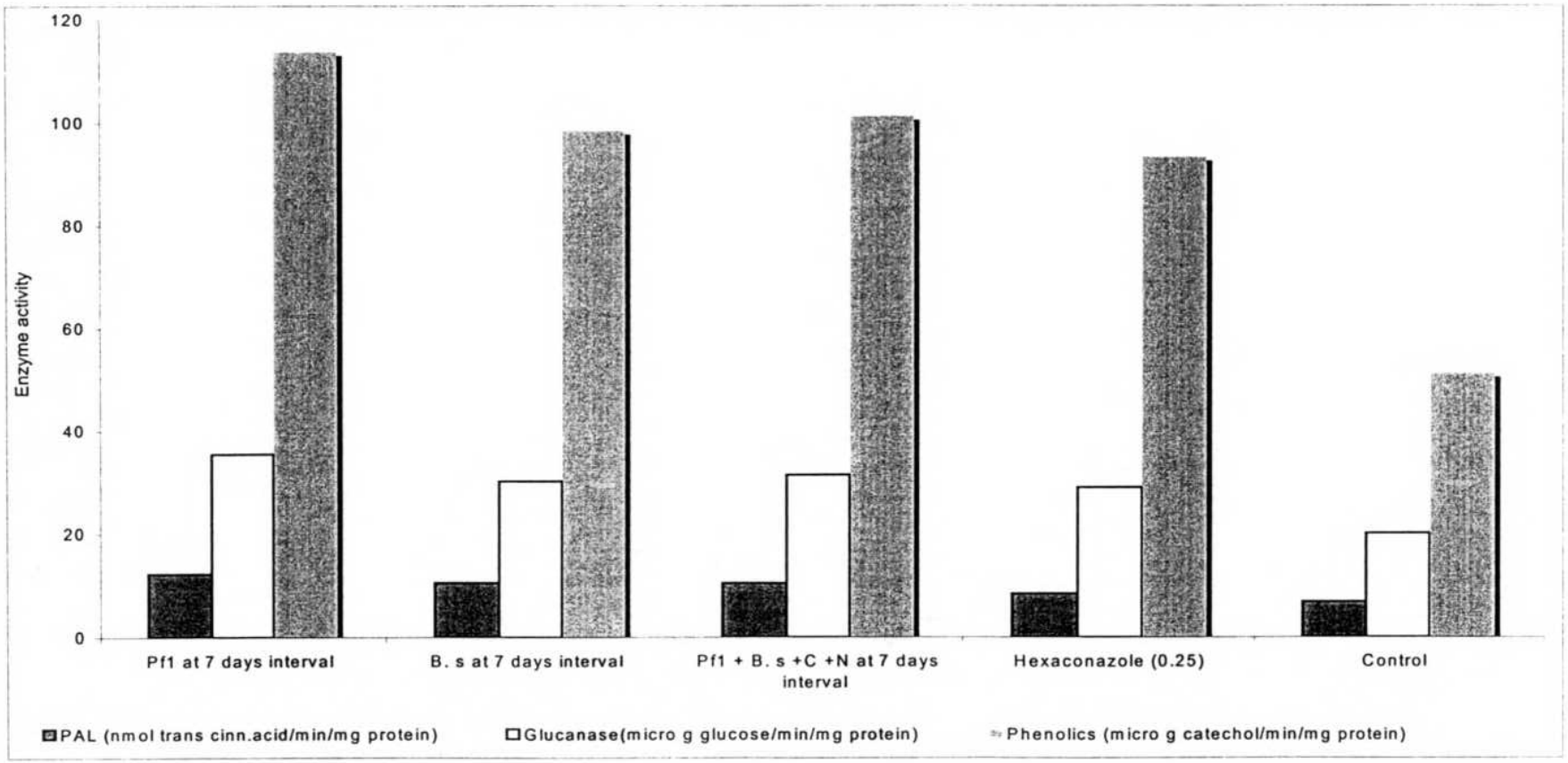
In a column, means followed by a common letter are not significantly different (p = 0.05) by DMRT

Fig 7. Induction of defense related enzymes in leaves treated with different PGPR bioformulations against blister blight of tea under field conditions



Pf1 - *Pseudomonas fluorescens*
B.s - *Bacillus subtilis*
C - Chitin
N - Neem

Fig 8. Induction of defense related enzymes in leaves treated with different PGPR bioformulations against blister blight of tea under field conditions



Pf1 - *Pseudomonas fluorescens*
B.s - *Bacillus subtilis*
C - Chitin
N - Neem

Plate 13. Field view of tea plantations at Anamalais Hills

Plate 13


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Plate 14a. Efficacy of *P. fluorescens* on disease incidence of blister blight of tea under field conditions

Plate 14b. Closer view of *P. fluorescens* bioformulation on disease incidence of blister blight of tea

Plate 14a

Plate 14b

Fig 9. Efficacy of different PGPR bioformulations against blister blight disease incidence in tea plantations under field conditions during 2001

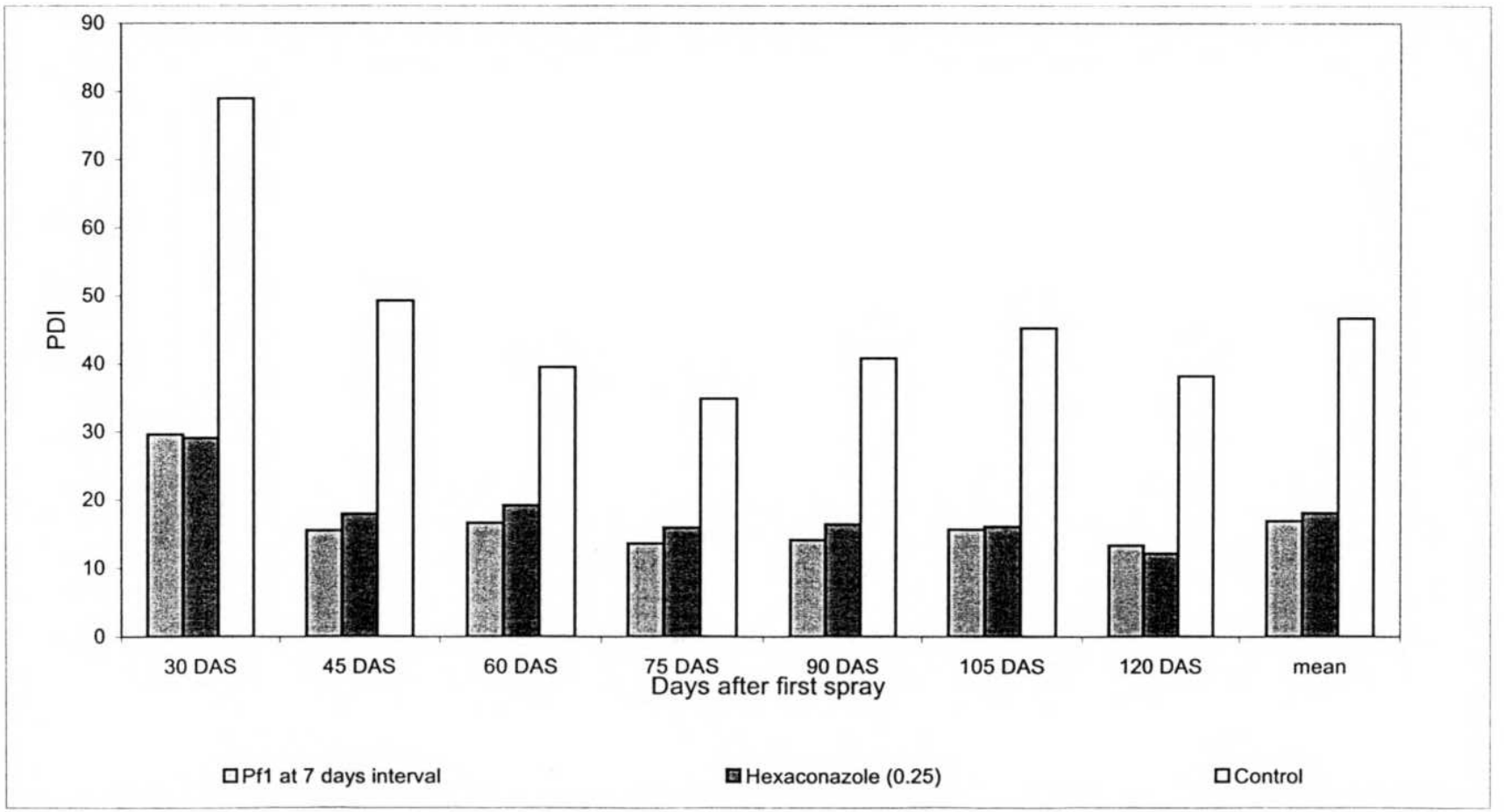


Table 21. Effect of foliar application of different PGPR mixtures on per cent disease index of blister blight in tea plantations under field conditions during 2001

Treatments	30 DAS	45 DAS	60 DAS	75 DAS	90 DAS	105 DAS	120 DAS	Mean
Pf-1 at 7 DI	29.62^{j}	15.54^{l}	16.63^{k}	13.58^{j}	14.13^{i}	15.66^{k}	13.33^{h}	16.92^{e}
	(32.96)	(23.18)	(26.04)	(21.58)	(24.04)	(23.28)	(21.37)	(24.26)
Pf-1 at 14 DI	37.84^{g}	26.26^{g}	23.10^{f}	18.86^{f}	21.05^{f}	24.22^{f}	19.63^{f}	25.85^{c}
	(37.95)	(28.13)	(27.32)	(25.71)	(27.29)	(29.46)	(26.28)	(30.54)
Pf-1 at 21DI	46.50^{e}	25.64^{d}	25.25^{d}	21.67^{d}	27.06^{d}	26.66^{d}	23.25^{d}	28.00^{bc}
	(42.99)	(30.48)	(30.15)	(27.72)	(31.33)	(31.07)	(28.81)	(31.93)
B. s at 7DI	33.18^{h}	18.00^{h}	20.66^{g}	17.32^{g}	21.02^{f}	21.22^{h}	17.62^{g}	21.28^{d}
	(35.16)	(25.08)	(27.01)	(24.57)	(27.27)	(27.41)	(24.79)	(27.45)
B. s at 14DI	49.91^{c}	24.63^{e}	25.88^{c}	21.00^{e}	24.65^{e}	25.99^{e}	23.50^{d}	28.43^{bc}
	(44.94)	(29.74)	(30.56)	(27.25)	(29.75)	(30.63)	(28.87)	(32.21)
B. s at 21DI	49.03^{b}	28.14^{e}	27.22^{b}	26.10^{c}	27.73^{c}	36.66^{b}	27.65^{b}	31.28^{b}
	(47.32)	(32.02)	(31.43)	(30.71)	(31.76)	(37.20)	(31.71)	(33.99)
Pf-1 + *B s* + C + N at 7DI	32.29^{i}	23.86^{f}	19.73^{i}	15.13^{i}	19.5^{g}	18.86^{i}	17.65^{g}	21.00^{d}
	(34.62)	(28.78)	(26.35)	(22.86)	(26.18)	(25.71)	(24.81)	(27.25)
Pf-1 + *B.s* + C + N at 14 DI	44.58^{f}	28.13^{b}	23.83^{e}	21.63^{d}	24.4^{e}	22.83^{g}	21.45^{e}	27.04^{c}
	(41.88)	(32.02)	(29.20)	(27.69)	(29.58)	(28.52)	(27.57)	(31.32)
Pf-1 + *B.s* + C + N at 21DI	48.14^{d}	30.60^{c}	26.38^{g}	23.77^{b}	28.93^{b}	27.31^{c}	24.59^{c}	29.60^{c}
	(43.93)	(33.57)	(26.81)	(31.14)	(32.52)	(31.49)	(29.71)	(30.78)
Hexaconazole (0.25)	29.10^{k}	17.94^{h}	19.20^{j}	15.91^{h}	16.42^{h}	16.08^{j}	12.18^{i}	18.11^{e}
	(32.63)	(25.03)	(25.96)	(23.48)	(23.87)	(23.61)	(20.38)	(25.16)
Control	79.00^{a}	49.33^{a}	39.54^{a}	34.90^{a}	40.80^{a}	45.25^{a}	38.25^{a}	46.72^{a}
	(62.74)	(44.61)	(38.95)	(36.20)	(39.69)	(42.27)	(38.20)	(43.11)

DI-days interval, C-chitin, N-neem, DAS-days after first spray

Values are mean of three replications, Values in parentheses are arcsine transformed

In a column, means followed by a common letter are not significantly different (p=0.05) by DMRT

Plate 15a. Efficacy of *B. subtilis* on disease incidence of blister blight of tea under field conditions

Plate 15b. Closer view of *B. subtilis* on disease incidence of blister blight of tea under field conditions

Plate 15a

Plate 15b

Plate 16a. Efficacy of PGPR bioformulations on disease incidence of blister blight of tea under field conditions

Plate 16b. Closer view of PGPR bioformulations on disease incidence of blister blight of tea under field conditions

Plate 16a

Plate 16b

Table 22. Effect of foliar application of different PGPR mixtures on per cent disease index of blister blight in tea plantations under field conditions during 2002

Treatments	Before pruning		After pruning	
	30 DAS	45 DAS	30 DAS	45 DAS
Pf-1 at 7 DI	27.59^{i} (31.61)	24.15^{j} (29.42)	13.25^{i} (21.20)	14.21^{i} (22.11)
Pf-1 at 14 DI	38.16^{g} (38.14)	35.16^{g} (36.36)	18.10^{f} (25.18)	19.92^{f} (26.48)
Pf-1 at 21DI	47.20^{e} (43.39)	44.29^{e} (41.71)	24.16^{d} (29.42)	28.18^{d} (32.05)
B. s at 7DI	34.15^{h} (37.75)	31.25^{h} (33.98)	16.26^{g} (23.15)	17.01^{g} (24.33)
B. s at 14DI	49.18^{c} (44.42)	46.15^{d} (42.86)	24.15^{d} (29.42)	23.25^{e} (28.81)
B s at 21DI	50.21^{b} (45.12)	51.15^{b} (45.65)	30.16^{b} (33.30)	30.01^{b} (33.20)
Pf-1 + *B.s* + C + N at 7DI	34.16^{h} (35.75)	29.08^{i} (32.62)	15.15^{h} (22.87)	16.16^{h} (23.67)
Pf-1 + *B.s* + C + N at 14 DI	45.10^{f} (42.18)	42.95^{f} (40 94)	22.01^{e} (27 96)	23 01^{e} (28.65)
Pf-1 + *B.s* + C + N at 21DI	48.10^{d} (43.91)	49.15^{c} (44.51)	27.25^{c} (31.45)	29.06^{c} (32.60)
Hexaconazole (0.25%)	26.50^{j} (30.97)	24.02^{j} (29.33)	12 75^{j} (20.88)	13.15^{j} (21.22)
Control	72.00^{a} (58.06)	69.15^{a} (56.26)	38.25^{a} (38.20)	41.25^{a} (39.95)

DI-days interval, C-chitin, N-neem, DAS-days after first spray

Values are mean of three replications, Values in parentheses are arcsine transformed

In a column, means followed by a common letter are not significantly different (p=0.05) by DMRT

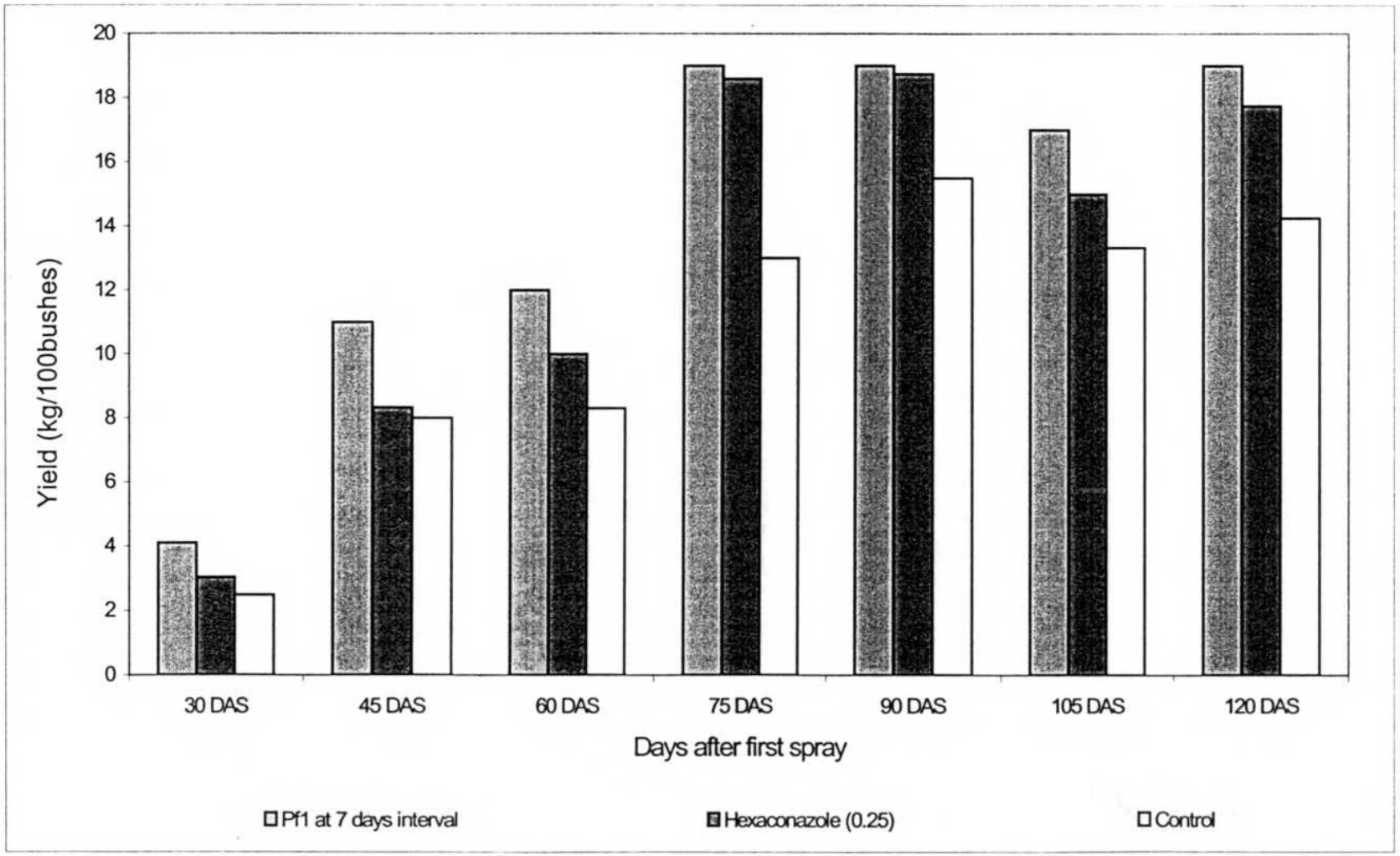
Plate 17a. Efficacy of fungicide on disease incidence of blister blight in tea plantations

Plate 17b. Closer view of effect of fungicide on disease incidence of blister blight in tea plantations

Plate 17a

Plate 17b

Fig 10. Effect of different PGPR bioformulations on yield of tea plantations under field conditions during 2001



2002 trial also. The results also showed that the yield increase by *P. fluorescens* at 14 days interval spray was on par with the weekly interval spray of *P. fluorescens* and *B. subtilis* combination with chitin and neem (Table 23, 24) (Plate 18, 19, 20).

Table 23. Efficacy of PGPR strains over the yield of tea plantations under field conditions during 2001

Treatments	30 DAS	45 DAS	60 DAS	75 DAS	90 DAS	105 DAS	120 DAS	Total yield (kg/100 bushes)
Pf-1 at 7 DI	4.10^{a}	11.00^{a}	12.00^{a}	19.00^{a}	19.00^{a}	17.00^{a}	19.00^{a}	101.10
Pf-1 at 14 DI	3.83^{bc}	10.00^{bc}	10.33^{b}	18.50^{b}	19.00^{a}	15.00^{b}	17.00^{b}	93.66
Pf-1 at 21DI	2.93^{c}	10.00^{bc}	9.66^{c}	18.50^{b}	18.00^{b}	14.00^{c}	15.00^{c}	88.09
B. s at 7DI	3.22^{b}	11.00^{b}	10.33^{b}	17.00^{b}	17.00^{b}	15.55^{b}	17.55^{b}	91.65
B. s at 14DI	2.92^{c}	10.00^{bc}	9.16^{c}	17.00^{b}	17.60^{c}	14.55^{c}	16.65^{c}	87.88
B. s at 21DI	2.85^{c}	10.00^{bc}	9.16^{c}	16.00^{c}	16.00^{d}	14.00^{c}	16.50^{c}	80.51
Pf-1 + *B s* + C + N at 7DI	3.23^{b}	9.00^{b}	11.33^{b}	18.60^{ab}	18.00^{b}	16.30^{b}	17.50^{b}	93.96
Pf-1 + *B s* + C + N at 14 DI	2.73^{d}	8.00^{d}	10.30^{b}	18.00^{b}	17.50^{b}	15.50^{b}	16.65^{c}	88.68
Pf-1 + *B s* + C + N at 21DI	2.44^{d}	8.00^{d}	9.60^{c}	14.30^{d}	15.00^{d}	14.50^{c}	15.25^{c}	79.09
Hexaconazole (0.25%)	3.03^{c}	8.33^{d}	10.00^{b}	18.60^{ab}	18.75^{a}	15.00^{b}	17.75^{b}	91.46
Control	2.48^{d}	8.00^{d}	8.30^{d}	13.00^{e}	15.50^{e}	13.33^{d}	14.25^{d}	77.86

DI-days interval, C-chitin, N-neem, DAS-days after first spray

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. Efficacy of PGPR strains over the yield (kg/ 100 bushes) of tea plantations under field conditions during 2002

Treatments	Before pruning		After pruning	
	30 DAS	45 DAS	30 DAS	45 DAS
Pf-1 at 7 DI	10.31^{h}	9.81^{d}	13.12^{e}	11.25^{d}
Pf-1 at 14 DI	8.85^{ef}	8.10^{bc}	11.98^{d}	9.83^{c}
Pf-1 at 21DI	7.65^{bc}	7.30^{a}	10.01^{c}	8.56^{b}
B. s at 7DI	9.05^{fg}	8.70^{b}	11.26^{cd}	9.98^{c}
B. s at 14DI	8.20^{cde}	7.90^{bc}	9.23^{bc}	8.79^{b}
B. s at 21DI	7.31^{bcd}	6.85^{a}	8.53^{a}	7.90^{a}
Pf-1 + *B.s* + C + N at 7DI	9.58^{g}	9.12^{c}	12.95^{d}	10.25^{c}
Pf-1 + *B.s* + C + N at 14 DI	8.56^{def}	7.94^{b}	10.94^{c}	8.90^{b}
Pf-1 + *B.s* + C + N at 21DI	7.40^{ab}	7.01^{a}	9.31^{bc}	8.12^{a}
Hexaconazole (0.25%)	8.90^{f}	8.30^{c}	9.12^{bc}	9.05^{bc}
Control	6.90^{a}	6.75^{a}	7.50^{a}	7.13^{a}

DI-days interval, C-chitin, N-neem, DAS-days after first spray

Values are mean of three replications

In a column, means followed by a common letter are not significantly different (p=0.05) by DMRT.

Plate 18a. Untreated control

Plate 18b. Closer view of untreated control

Plate 18a

Plate 18b

Plate 19. Efficacy of *P. fluorescens* on growth promotion of tea leaves

1. **Control**
2. **Fungicide**
3. **Pf1 (7 days interval)**
4. **B (7 days interval)**
5. **Pf1 + B + Chitin + Neem (7 days interval)**

Plate 19

Plate 20. Efficacy of *P. fluorescens* on yield of tea plantations

Plate 20

Discussion

CHAPTER V

DISCUSSION

Exobasidium vexans Massee and *Macrophomina phaseolina* (Tassi) Goid are the most important biotroph and necrotroph pathogens causing severe loss to the economy of tea industry and green gram crop respectively. Management of these diseases with cultural methods and toxic chemicals has it's own merits and demerits. Management of these diseases with nonchemical methods has gained importance nowadays. Recent quests for effective, safe and lasting disease management programs have been targeted primarily towards the development of new and better products with which to replace conventional toxic pesticides.

Induced systemic resistance is a promising defense tool in plant protection. This is based on the activation of plant defense mechanisms by PGPR strains. It is considered natural, ecofriendly and safe, provides resistance against a broad spectrum of pathogens (Sticher *et al.*, 1997). Hence, utility of PGPR strains as promising biocontrol agents against the blister blight of tea and dry root of green gram is focussed here.

The development of biological techniques using PGPR strains is an emerging arena in crop protection to reduce the damage to economic crops caused by plant pathogens. Many PGPR stains have been isolated from rhizosphere soil of different crops, soil, irrigation water and from plants in different countries by several workers and tested against several diseases (Nandakumar *et al.*, 2001; Viswanathan, 1999; Rabindran and Vidhyasekaran, 1996; Rosales *et al.*, 1993; Yuen *et al.*, 2001; Gupta *et al.*, 2002). This has resulted in the commercialization of a number of microbial biocontrol products especially talc based formulations (Fravel and Larkin, 1996; Vidhyasekaran and Muthamilan, 1999, Nandakumar *et al.*, 2001). Multiple strain mixture of microbial agents has been employed with some success against several plant pathogens in previous

studies. These include mixture of fungi (Paulitz *et al.*, 1990; Budge *et al.*, 1995; Schisler *et al.*, 1997), mixture of bacteria (Pierson and Weller, 1994; Raupach and Kloepper, 1998; Singh *et al.*, 1999), mixture of yeasts (Janisiewicz, 1996), bacteria and fungi (Duffy *et al.*, 1996; Leibinger *et al.*, 1997) and bacteria and yeast (Janisiewicz and Bors, 1995). With this knowledge, talc-based formulations containing either single or mixture of PGPR strains amended with or without chitin were tested against the biotroph (*Exobasidium vexans*) and necrotroph (*Macrophomina phaseolina*) pathogens under greenhouse and field conditions.

5.1. *In vitro* screening of the biocontrol agents against *M. phaseolina*

The mycoparasitic potential of *Pseudomonas* spp. is well documented (Keel and Defago, 1997; Whipps, 1997). This phenomenon has often been used as means for *in vitro* screening of biocontrol agents (Hadar *et al.*, 1979; Elad *et al.*, 1980). In the present study, *P. fluorescens* Pf1 showed maximum mycelial inhibition of *M. phaseolina.*

Production of antibiotics *viz.*, HCN, pyrrolnitrin, phenazine and 2,4-diacetyl phloroglucinol and lytic enzymes by *P. fluorescens* against fungal pathogens were reported by many workers (Ramamoorthy and Samiyappan, 2001; Viswanathan and Samiyappan, 2001b).

The above facts suggest that the inhibition of root rot pathogen, *M. phaseolina* by *P. fluorescens* Pf1 may be due to the production of antibiotics, siderophore mediated competition and lytic enzymes *viz.*, chitinase, β-1, 3-glucanase which might have degraded the fungal cell wall and restricted the growth of fungus under *in vitro* conditions.

5.2. Efficacy of PGPR formulations against dry root rot disease in green gram

5.2.1. Greenhouse experiments

PGPR strains which performed well in both plant growth promotion studies and in reducing the mycelial growth of *M. phaseolina* were selected and used against the dry root rot pathogen under greenhouse condtions in different combinations amended with or

without chitin and neem. In addition to these combinations, mixture of two PGPR strains was also tried to test the efficacy of the bioformulations against the dry root rot disease in green gram. PGPR strain, *P. fluorescens* Pf1 along with chitin was found to be effective in significantly reducing the disease incidence of root rot under greenhouse conditions than the application of *P. fluorescens* Pf1 strain without chitin and untreated control.

Application of PGPR strains alone or in combination with chitin towards managing plant diseases has been demonstrated in few crops. Unique biological properties of chitin oligomers including their antifungal properties on various plant pathogenic fungi such as *Fusarium oxysporum* f.sp. *radicis-lycopersici* and *Pythium aphanidermatum* have been well documented (Lafontaine and Benhamou, 1996; El Ghaouth *et al.,* 1994; Benhamou, 1992; Leuba and Stossel, 1986). The chitin oligomers were also reported as potential elicitors of plant defense reactions (Benhamou, 1992; Leuba and Stossel, 1986).

Chitin has got wide applications against various fungal pathogens and this justifies its use as an amendment in bioformulation mixture in the present study. Chitin amendment increased the growth and multiplication of chitinolytic microflora (Culbreath *et al.,* 1986; Bell *et al.,* 1998). Viswanathan and Samiyappan (2001a) reported that when chitin was substituted with glycerol as a carbon source, it resulted in enhanced growth and multiplication of pseudomonads. Yuen *et al.* (2001) also found that incorporation of chitin in the medium increased bacterial population when compared to the non-chitin amended medium and improved the efficiency of PGPR strains in reducing the severity of rust disease in bean plants.

Similarly, foliar application of chitin to peanut were reported to enhance biocontrol of early leaf spot with a chitinolytic strain, *B. cereus* by providing a nutrient source for the applied bacterium and resident chitinolytic microbes (Kokalis-Burelle *et al.*, 1992).

The reduction in dry root rot by *P. fluorescens* Pf1 was probably attributed to action of Induced Systemic Resistance (ISR) or Systemic Acquired Resistance (SAR) mechanisms or combination of both.

5.3. Effect of PGPR strains on plant growth promotion.

5.3.1. Seed treatment followed by soil application

Seed treatment followed by soil application of PGPR formulations especially talc based formulations of *P. fluorescens* Pf1 along with chitin were found to increase plant growth of green gram seedlings both under greenhouse and field conditions.

Many rhizobacteria have been reported to stimulate plant growth (Schroth and Hancock, 1982; Ramamoorthy *et al.*, 2002b). Group of *Pseudomonas* spp. significantly improved emergence of seedlings and the proportion of healthy seedlings in sugar beet when compared to seedlings from untreated seeds (Williams and Asher, 1996). Seed treatment with fluorescent pseudomonads increased plant growth promotion in tomato and hotpepper (Ramamoorthy *et al.*, 2002a).

An increase in germination of 30 to 60 per cent was observed in maize by seed treatment with PGPR strains *P. aeruginosa* NSK2 and *P. fluorescens* ANP 15 (Hofte *et al.*, 1991). Seed and soil application of PGPR strains showed plant growth promotion with increased runner length and increased leaf number per plant in cucumber (Wei *et al.*, 1996). Seed, root, foliar and soil application of PGPR strains promoted plant growth in terms of increased plant height and number of tillers and ultimately grain yield in rice correlated with sheath blight disease reduction (Nandakumar *et al.*, 2001).

Seed bacterization by fluorescent pseudomonads proved to be a potential method for the enhancement of plant growth and suppression of *Rhizoctonia solani* and *Sclerotium rolfsii* in peanut seedlings (Dube and Podile, 1989). The positive colonization ability of *Pseudomonas* GRC_2 lies in it being the successful colonizer of the spermosphere, increased seedling emergence and its establishment in the rhizosphere of peanut giving protection against *M. phaseolina* resulting in enhanced yield. The presence

of a hydroxamate type of siderophore produced by *Pseudomonas* GRC not only helped in biocontrol but also improved overall plant health as well due to alleviation of iron-based chlorosis (Gupta *et al.*, 2002). Root colonization by fluorescent pseudomonads protected the peanut seedlings from *F. solani* (Anderson and Guerra, 1985).

Pseudomonas spp. has been very well known for its IAA producing ability, which is effective for plant growth promotion (Sivamani and Gnanamanickam, 1988; Kumar Dileep and Dube, 1992; O'Sullivan and O'Gara, 1992).

PGPR strains INR 7 (*Bacillus pumilis*), GBO 3 (*B. subtilis*) and also *Curtobacterium flaccumfaciens* applied individually or in combination as seed treatment showed intensive plant growth promotion correlated with reduction of multiple pathogens *viz., Colletotrichum orbiculare, P. syringae* pv. *lachrymans* and *Erwinia tracheiphila* in cucumber (Raupach and Kloepper, 1998).

PGPR increase plant growth directly by mediating the production of secondary metabolites and phytohormones such as auxins, cytokinins or gibberellic acid (Arshad and Frankenberger, 1991; Beyeler *et al.*, 1999) or indirectly either by suppression of well-known diseases caused by major pathogens or by reducing the deleterious effects of minor pathogens (Schippers *et al.*, 1987).

Alternatively PGPR may increase plant growth by association with N_2 fixation (Hong *et al.*, 1991), solubilizing nutrients such as P (Whitelaw, 2000), promoting mycorrhizal function (Garbaye, 1994), regulating ethylene production in roots (Glick, 1995) and decreasing heavy metal toxicity (Burd *et al.*, 1998). Hence plant growth promotion by PGPR strains especially *P. fluorescens* Pf1 in green gram seedlings may be due to the induction of plant growth hormones and suppression of deleterious pathogens or combination of both from the rhizosphere and spermosphere.

5.3.2. Foliar application

From the field study conducted at Anamalais hills, the foliar application of talc based formulations of PGPR strains were found to increase the yield of tea plantations during both seasons (2001 and 2002).

Similar findings were reported by Reddy *et al.* (2001) that the foliar spray of powder formulation of PGPR strains promoted the plant growth besides effectively controlling the tomato bacterial spot (*Xanthomonas axonopodis* pv. *vesicatoria*), cucumber angular leaf spot (*P. syringae* pv. *lachrymans*), tobacco blue mold (*Peronospora tabacina*) and wild fire (*P. syringae* pv. *tabaci*).

5.4. Expression of defense related proteins and chemicals by PGPR formulations against *M. phaseolina*

Enhancement of defensive capacity of the plants against broad spectrum of pathogens and pests is acquired after appropriate stimulation. The resulting elevated resistance due to biotic agents (eg. PGPR) is referred to as ISR where as that by other than biocontrol agents is called SAR (van Loon *et al.*, 1998). In our study, we concentrated on biotic (biocontrol agents) and abiotic (chitin) inducers for inducing the defense molecules against the necrotroph pathogen, *M. phaseolina.* The acquired resistance in plants was mainly focussed in this study by the induction of defense related proteins and chemicals *viz.,* Phenylalanine ammonia lyase (PAL), Peroxidase (PO), Polyphenol oxidase (PPO), phenols, chitinase and β-1, 3-glucanase.

5.4.1. Induction of enzymes in phenyl propanoid pathway

Phenylalanine ammonia lyase (PAL) plays an important role in the biosynthesis of various defense chemicals in phenyl propanoid metabolism (Daayf *et al.*, 1997). PAL activity could be induced in plant-pathogen interactions and fungal elicitor treatment (Ramanathan *et al.*, 2000). de Meyer *et al.* (1999) reported that rhizosphere colonization of *P. aeruginosa* 7 NSK 2 activated PAL in bean roots and increased the salicylic acid levels in leaves. In the present study, increased activity of PAL was recorded in the plants

treated with bioformulation containing PGPR strain Pf1 along with chitin and challenge inoculated with fungal pathogen. The timing and expression patterns of the defense mechanisms are important for the suppression of pathogen.

Higher level expression of defense related proteins and timely accumulation of chemicals at the infection site certainly prevent the colonization of pathogen in green gram seedlings treated with the bioformulation amended with chitin.

The maximum accumulation of PAL at 21 days of challenge inoculation coincides with the time which is normally favourable for the pathogen to infect green gram plants. The increased activity of PAL may also be constituted for enhancing the resistance in green gram plants against fungal pathogen, *M. phaseolina*.

Induction of PAL by fluorescent pseudomonads was reported in cucumber against *P. aphanidermatum* (Chen *et al.*, 2000), tomato against *F.oxysporum* f. sp. *lycopersici* (Ramamoorthy *et al.*, 2002b) and bean against *Botrytis cinerea* (Zdor and Anderson, 1992).

5.4.2. Peroxidase

Increased PO activity has been shown in a number of resistant interactions involving plant pathogenic fungi, bacteria and viruses (Chen *et al.*, 2000; Nandakumar *et al.*, 2001; Dalisay and Kuc, 1995; Kandan *et al.*, 2002). Peroxidases have been implicated in a number of physiological functions that may contribute to resistance including exudation of hydroxy-cinnamyl alcohol into free radical intermediates (Gross, 1980), phenol oxidation (Schmidt and Feucht, 1980), polysaccharide cross linking (Fry, 1986), cross linking of extensin monomers (Everdeen *et al.*, 1988) and lignification (Walter, 1992) and also associated with deposition of phenolic compounds into plant cell walls during resistant interactions (Graham and Graham, 1991). Accumulation of peroxidase has been correlated with induced systemic resistance in several plants (Ramamoorthy and Samiyappan, 2001; Chen *et al.*, 2000; Dalisay and Kuc, 1995; Hammerschmidt *et al.*, 1982).

In the present study, green gram plants treated with the bioformulation containing *P. fluorescens* Pf1 amended with chitin and challenged with the pathogen showed higher induction of peroxidase.

Induction of four isozymes *viz.*, PO1, PO2, PO3 and PO4 in treatments with the bioformulation of *P. fluorescens* Pf1 along with chitin and challenge inoculated with *M. phaseolina* was observed. The induction of isoforms was more prominent in the treatments involving bioformulation with chitin than without chitin.

5.4.3. Polyphenol oxidase

The present study also indicated that the application of the bioformulation mixture along with chitin induced PPO. Induction was higher in plants treated with bioformulation amended with chitin and challenged with the pathogen.

Three isoforms (PPO1, PPO2 and PPO3) were induced in plants treated with the PGPR formulation mixture amended with chitin and challenge inoculated with *M. phaseolina* and they were prominently expressed when compared to untreated control.

Earlier, Radja Commare (2000) reported that *P. fluorescens* Pf1 induced PPO isozymes in rice against *R. solani*. Chen *et al.* (2000) reported that various rhizobacteria and *P. aphanidermatum* induced PPO activity in cucumber root tissues.

5.4.4. Phenolics

Phenolic compounds enhance the mechanical strength of host cell wall and also inhibit the invading pathogenic organisms. Seed treatment with *P. fluorescens* 63 induced the accumulation of phenolics in tomato root tissue (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.*, 1996a).

The present study also indicated that the higher level of accumulation of phenolics occurred in green gram plants treated with Pf1 along with chitin and challenge inoculated

with the fungal pathogen. Similar findings were reported in sugarcane against *C. falcatum* (Viswanathan and Samiyappan, 1999), in tomato and hotpepper against *P. aphanidermatum* (Ramamoorthy *et al.*, 2002a) and in rice against *R. solani* (Radja Commare, 2000). Benhamou *et al.* (2000) reported that an endophytic bacterium, *Serratia plymuthica* induced the accumulation of phenolics in cucumber roots against *P. ultimum.*

5.4.5. Chitinase and β-1, 3-glucanase

Pathogenesis related (PR) proteins are host coded proteins induced by different types of pathogens and abiotic stresses (van Loon, 1997). Synthesis and accumulation of PR proteins have been reported to play an important role in plant defense mechanisms. Chitinases and β-1, 3-glucanases (which are classified under PR-3 and PR-2 groups of PR proteins respectively) have been reported to associate with plant disease resistance (Maurhofer *et al.*, 1994; van Loon, 1997).

In general, fungal cells contain chitin and glucan as their cell wall constituents. The main mode of antagonistic activity of microbes is production of lytic enzymes (chitinases and β-1, 3-glucanases) which act on cell walls of organisms which have chitin and glucan as their cell wall component (Singh *et al.*, 1999) and also through induced systemic resistance (ISR) in plant system.

In the present study, it was noticed that the elevated levels of chitinase and β-1, 3-glucanase in plants treated with bioformulation containing Pf1 along with chitin and challenge inoculated with pathogen. Two new chitinase isoforms with molecular weight of 40 (chi 1) and 45 (chi 2) kDa were detected in Western blot analysis from the plants treated with bioformulation of Pf1 strain along with chitin and challenge inoculated with *M. phaseolina.*

Similarly, Ramamoorthy *et al.* (2002b) reported the induction of 45 and 46 kDa chitinase isoforms in *P. fluorescens* treated tomato plants challenged with *F. oxysporum* f.sp. *lycospersici.* In tobacco, induction of two PR proteins *viz.*, β-1, 3-glucanase and

chitinase were noticed due to application of *P. fluorescens* isolate CHAO in response to infection by tobacco necrosis virus (TNV) (Maurhofer *et al.*, 1994). Induction of hydrolytic enzymes was also reported in pea against *P. ultimum* and *F. oxysporum* f.sp. *pisi* (Benhamou *et al.*, 1996a, b). The enzymatic degradation of the fungal cell wall by hydrolytic enzymes may release non-specific elicitors (Ham *et al.*, 1991) which in turn elicit various defense reactions. The fungal cell wall elicitors have been reported to elicit various defense reactions in green gram (Ramanathan *et al.*, 2000). Viswanathan and Samiyappan (1999) reported that ISR by fluorescent pseudomonads which was associated with induction of chitinase appear to be the promising technology for the management of red rot disease of sugarcane.

From the above results, it is concluded that the induction of defense related proteins and chemicals by PGPR strain Pf1 along with chitin bioformulation might be used as promising tool for the management of root rot pathogen in green gram. Timely and enhanced induction of such proteins and defense mechanisms upon pretreatment with PGPR strain Pf1 might have prevented the colonization of the fungal pathogens in the plant system under green house conditions.

5.5. Field experiments

5.5.1. Rhizosphere colonization

From field trials, rhizosphere colonization ability of P. *fluorescens* Pf1 with or without chitin and neem was studied. *P. fluorescens* Pf1 along with chitin amendment was found to be the effective for survival and colonization of bacteria under field conditions. The colonization ability of Pf1 may be correlated with its competitive ability against the deleterious organisms thereby inhibiting the colonization of pathogenic organisms around the plant system. Root colonization by rhizobacteria appears to be an important factor in plant growth promotion and biological control (de Weger *et al.*, 1995; Knudsen *et al.*, 1997; Roberts *et al.*, 1999).

Jayashree *et al.* (2000) reported that, disease suppression by fluorescent pseudomonads depends on its ability to colonize rhizosphere. The colonization of roots by inoculated bacteria is an important step in the interaction between beneficial bacteria and the host plant to reduce the attack of deleterious pathogen (Benizri *et al.*, 2001). Effective colonization of rhizosphere by the application of Pf1 strain along with chitin significantly reduced disease incidence of root rot and increased the yield in green gram under field conditions.

5.5.2. Efficacy of *P. fluorescens* against dry root rot disease in green gram

PGPR strain *P. fluorescens* Pf1 along with chitin mixture exhibiting the better results under green house conditions were taken into account and different combinations of the strain with or without chitin and neem were used in the field study.

In field experiments, the talc based bioformulation mixture containing Pf1 along with chitin showed significantly less disease incidence and correspondingly resulted in enhanced yield. Similarly, talc based formulation was found to be effective in controlling chick pea wilt (Vidhayasekaran and Muthamilan, 1995), pigeon pea wilt (Vidhyasekaran *et al.*, 1997b), rice sheath blight (Vidhyasekaran and Muthamilan, 1999; Radja Commare, 2000; Nandakumar *et al.*, 2001) and red rot of sugarcane (Viswanathan and Samiyappan, 2001a). In addition to disease control, strain mixtures enhanced the plant growth in terms of increased seedling emergence (Dunne *et al.*, 1998; Pierson and Weller, 1994; Duffy *et al.*, 1996). Formulation containing *B. subtilis* and *B. amyloliquefaciens* and chitosan as an elicitor was found to be effective against several soil pathogens in tomato crop (Reddy *et al.*, 1999).

ISR against several plant pathogens by fluorescent pseudomonads was reported under field conditions (Wei *et al.*, 1991; Vidhyasekaran and Muthamilan, 1999; Nandakumar *et al.*, 2001; Zehnder *et al.*, 2001). From the present study also, it is clearly established that bioformulation containing fluorescent pseudomonads mediated

sustainable protection of green gram under field conditions through ISR against root rot disease with simultaneous enhancement in yield.

5.6. Effect of PGPR strains against blister blight in tea plantations

5.6.1. Field experiments

Since the blister blight pathogen is biotrophic in nature, *in vitro* studies could not be carried out for screening biocontrol agents. Hence, the talc based formulations of PGPR strains based on their earlier performances on several crops were selected and used against the blister blight of tea with different combinations under field conditions. The consistency of PGPR strains was tested for two years and from the study, it was found that PGPR strain *P. fluorescens* Pf1 bioformulation produced better results in reducing the disease incidence of blister blight of tea at Anamalais hills when compared to untreated control and also it was comparable with chemical treatment.

Most of the studies carried out directly under field conditions using PGPR strains against the biotroph pathogens were reported by several workers (Umesha *et al.*, 1998; Singh *et al.*, 1999; Dagmar *et al.*, 1989).

Similar findings were reported by Enebak and Carey, (2000) that PGPR strains reduced the infection of an obligate pathogen on a pine host and indicated that PGPR-mediated systemic protection occurs for fusiform rust. PGPR treatment not only reduced the visual symptoms and pathogen development of *Peronospora tabacina* in plants, but also reduced sporulation. This suggests that PGPR mediated ISR may affect the efficiency of sporulation, an important event of an obligate biotroph's life cycle. Also, Northern analysis using four probes derived from cDNA clones of plant defense related genes, including the salicylic acid inducible PR-1a gene, suggested that ISR mediated by the PGPR strains may be associated with novel defense pathways (Zhang *et al.*, 2000).

From this, it is assumed that PGPR strain Pf1 mediated ISR may play a vital role in reducing the incidence of blister blight of tea under field conditions.

5.6.2. Induction of defense chemicals

Defense related enzymes were assayed from the tea plantations treated different combinations of PGPR strains sprayed at different days interval. The defense related enzymes *viz.*, phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, phenolics, chitinase and β-1, 3-glucanase accumulation were found to be more in tea plants treated with Pf1 bioformulation at weekly interval than untreated control. When groundnut plants were sprayed with *P. fluorescens*, increased activity of PAL was observed (Meena *et al.*, 2000).

The defense chemicals induced upon treatment with bioformulations might have reduced the disease incidence and thereby simultaneous increase in yield would have been recorded.

From testing the efficacy of PGPR strains against biotroph and necrotroph pathogens in tea and green gram, it is elucidated that *P. fluorescens* Pf1 bioformulation along with chitin was effective against dry root rot and *P. fluorescens* Pf1 bioformulation alone reduced the blister blight significantly under field conditions. PGPR mediated ISR against biotroph and necrotroph pathogens may become the efficient tool for the management of blister blight of tea and dry root rot of green gram.

In future, PGPR bioformulations may commercialized and tested for the wide range of biotroph and necrotroph pathogens of crop plants. The defined role of PGPR in plant defense mechanisms against the biotroph and necrotroph pathogens both under green house and field conditions are to be exploited intensively by having this manuscript as baseline.

Summary

CHAPTER VI
SUMMARY

1. The present study was under taken to assess the PGPR mediated induced systemic resistance against root rot in green gram under greenhouse and field conditions and blister blight in tea under field conditions. PGPR strains increased the germination capacity of green gram seeds, shoot and root length of seedlings and ultimately vigour index.

2. PGPR strain *P. fluorescens* Pf1 inhibited the mycelial growth of *Macrophomina phaseolina* under *in vitro* conditions.

3. Seed treatment followed by soil application of *P. fluorescens* along with chitin bioformulation significantly reduced the disease incidence of dry root rot and ultimately increased the yield of green gram under greenhouse conditions when compared to untreated control.

4. Increase in the activity of defense enzymes *viz.*, phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, chitinase, β-1, 3-glucanase and phenolics content were found to be significant in *P. fluorescens* along with chitin treated green gram plants challenged with pathogen when compared to control.

5. Green gram plants treated with *P. fluorescens* Pf1 along with chitin bioformulation expressed four isoforms of peroxidase *viz.*, PO1, PO2, PO3 and PO4 and three isoforms of polyphenol oxidase *viz.*, PPO1, PPO2 and PPO3 upon pathogen inoculation.

6. Among the various combinations of *P. fluorescens* along with or without chitin and neem used, green gram plants treated with *P. fluorescens* Pf1 along with chitin mixture showed less disease incidence than the untreated control under field conditions.

7. Application of *P. fluorescens* amended with chitin medium increased the survival ability and rhizosphere colonization of Pf1 in the of green gram plants under field conditions.

8. From field study conducted at Anamalais hills, foliar spray of *P. fluorescens* (@ 5g/litre of water) at weekly interval significantly reduced the disease incidence of blister blight in tea during 2001 and 2002 seasons under field conditions when compared to untreated control and the efficacy of PGPR bioformulation was comparable with chemical treatment.

9. Tea plants treated with *P. fluorescens* at weekly interval, significantly increased the activity of defense enzymes *viz.*, phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, chitinase, β-1, 3-glucanase and phenolics content under field conditions.

10. Weekly spray of *P. fluorescens* Pf1 significantly increased the yield in tea plantations when compared to untreated control and chemical treatment.

11. From all the aspects of study conducted in both green gram and tea using PGPR strains, *P. fluorescens* Pf1 along with chitin formulation performed better in reducing the root rot disease in green gram and talc based formulation of *P. fluorescens* as foliar spray at weekly interval was effective in reducing the disease intensity of blister blight of tea.

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* Original are not seen

Appendix

ANNEXURE I

King's B Medium (KB)

Peptone	: 20.0 g
Dipotassium hydrogen phosphate	: 1.5 g
Magnesium sulphate	: 1.5 g
Glycerol	: 10.0 g
Agar	: 15.0 g
Distilled water	: 1000 ml

ANNEXURE II

Nutrient Agar Medium (NA)

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

ANNEXURE III

REAGENTS USED FOR CHITINASE ASSAY

Preparation of colloidal chitin

Colloidal chitin for chitinase assay was prepared from crab shell chitin (Sigma, USA) by the method described by Berger and Reynolds (1958). The chitin (1g) was mixed with acetone to form paste. Then it was ground in a mortar by slowly adding 5-10 volumes of conc. HCl while maintaining the temperature at 5-10°C. After grinding, the material was filtered through glass wool and poured into 50% aqueous ethanol with vigorous stirring to precipitate the chitin in a highly dispersed state. The colloid was sedimented and resuspended in water several times to remove excess acid and ethanol and then dialyzed against distilled water. Chitin content was determined by drying the sample in vaccum and adjusted with distilled water to get a final concentration of 10 mg/ml (dry w/v) and stored at 4°C.

Snail gut enzyme (3%)

300 mg desalted snail gut enzyme (Helicase) in 10 ml distilled water (Boller *et al.*, 1983).

Para dimethyl aminobenzaldehyde (DMAB) reagent

8 g DMAB in 70 ml glacial acetic acid and 10 ml conc. HCl (10 N). It was diluted to 9 times with glacial acetic acid just before use (Reissig *et al.*, 1995).

Potassium tetra borate buffer (0.8 M, pH 9.2)

Aqueous solution of potassium tetra borate (0.8 M) was prepared and pH was adjusted to 9.2 with KOH.

ANNEXURE IV

SDS-PAGE

Acrylamide stock

Acrylamide	: 30 g
Bisacrylamide	: 0.8 g
Distilled water	: 100 ml

Separating gel (12%) 7.5 ml

Acrylamide stock solution	: 3.00 ml
Distilled water	: 2.50 ml
1.5 M Tris buffer, pH 8.8	: 1.875 ml
10 % SDS (Sodium dodecyl sulphate): 75 µl	
10 % APS (Ammonium per sulphate): 38 µl	
TEMED (N, N, N', N'-tetra methyl ethylene diamine)	: 5µl

Stacking gel (4 %) 5.0 ml

Acrylamide stock solution	: 0.65 ml
Distilled water	: 3.25 ml
1.5 M Tris buffer, pH 6.8	: 1.25 ml
10 % SDS (Sodium dodecyl sulphate): 50µl	
10 % APS (Ammonium per sulphate): 25 µl	
TEMED (N, N, N', N'-tetra methyl ethylene diamine)	: 5 µl

Sample buffer

20 % (v/v) Glycerol

0.125 M Tris buffer, pH 6.8

5 mM Na_2 EDTA

20 % (w/v) SDS

0.1 % (w/v) Bromophenol blue

1 % (v/v) 2-Mercaptoethanol

Electrode buffer

Glycine	: 4.320 g
Tris base	: 900 mg
SDS	: 300 mg

(Contents dissolved in 200 ml distilled water and the volume was made upto 300 ml with distilled water)

Staining solution

Coomassie brilliant blue R-250	: 200 mg
Methanol	: 40 ml
Glacial acetic acid	: 10 ml
Distilled water	: 50 ml

Destaining solution

Methanol	: 40 ml
Glacial acetic acid	: 10 ml
Distilled water	: 50 ml

ANNEXURE V

WESTERN BLOTTING

Anode buffer I

Tris base	:18.17 g
Methanol	:100 ml
Distilled water	:350 ml

(Contents dissolved in 200 ml distilled water, pH adjusted to 10.4 with NaOH and the volume was made upto 500 ml with distilled water)

Anode buffer II

Tris base	:1.51 g
Methanol	:100 ml
Distilled water	:500 ml

(Contents dissolved in 200 ml distilled water, pH adjusted to 10.4 with NaOH and the volume was made upto 500 ml with distilled water)

Cathode buffer I

Tris base	:1.51 g
6-amino hexanic acid	:2.62 g
Methanol	:100 ml
Distilled water	:500 ml

(Contents dissolved in 200 ml distilled water, pH adjusted to 9.4 with NaOH and the volume was made upto 500 ml with distilled water)

Tris buffered saline (TBS):10x stock

100 mM Tris base	:6.05 g
1.4 M NaCl	:40.91 g
Distilled water	:500 ml

(Contents dissolved in 200 ml distilled water, pH adjusted to 7.9 with Hcl and the volume was made upto 500 ml with distilled water)

Tris buffered saline – Tween (TBS-T):1x stock

10 x TBS stock	:100 ml
Tween 20	:0.5 ml
Distilled water	:899.5 ml

ANNEXURE VI

Native-gel electrophoresis

Separating gel (8 %) 7.5 ml

Acrylamide stock solution	: 2.00 ml
Distilled water	: 3.625 ml
1.5 M Tris buffer, pH 8.8	: 1.875 ml
10 % SDS (Sodium dodecyl sulphate)	: 75 µl
10 % APS (Ammonium per sulphate)	: 25 µl
TEMED (N, N, N', N'-tetra methyl ethylene diamine)	: 5µl

Soaking buffer

Ammonium bicarbonate	: 3.16 g
Distilled water	: 100 ml

Elution buffer

Ammonium bicarbonate	: 1.98 g
Distilled water	: 500 ml