

***PSEUDOMONAS FLUORESCENS* MEDIATED SYSTEMIC RESISTANCE IN
RICE AGAINST SHEATH BLIGHT DISEASE AND LEAFFOLDER INSECT**

Thesis submitted in part fulfillment of the requirements for the degree of **MASTER OF
SCIENCE (AGRICULTURE)** in **PLANT PATHOLOGY** to the
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By

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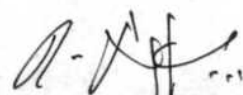
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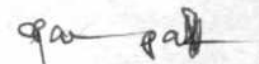
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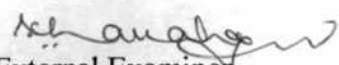
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Abstract

ABSTRACT

***PSEUDOMONAS FLUORESCENS* MEDIATED SYSTEMIC RESISTANCE IN RICE AGAINST SHEATH BLIGHT DISEASE AND LEAFFOLDER INSECT**

by

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The sheath blight pathogen, *Rhizoctonia solani* and the leaffolder, *Cnaphalocrocis medinalis* are the major yield constraints in rice crop. The effect of talc-based formulation of two *Pseudomonas fluorescens* strains (PF1 and FP7) and its mixture (with and without chitin) in inducing systemic resistance was tested against sheath blight and leaffolder incidence in rice. The application of talc-formulation through seed, root, soil and foliar spray significantly reduced the sheath blight and leaf folder incidence both under greenhouse and in two field experiments. The mixture of two strains performed better than their individual strains in all the experiments. The addition of chitin in the formulation further reduced the pest and disease incidence. Maximum of 62.0%

and 62.1% sheath blight and 47.7% and 56.1% reduction in leaffolder incidence was noticed in the mixture treatment containing chitin in two field trials respectively and its efficacy was statistically equivalent to standard fungicide and insecticide treatment. Moreover increased population of natural enemies of leaffolder viz., hymenopterans and spider were noticed in *Pseudomonas* treated plots under field conditions. In addition to pests and disease control, the *Pseudomonas* treatment consistently increased the yield in both the trials. Maximum of 12.0% and 21.1% increased yield was observed in two trials respectively. In the *in vitro* leaf folder bioassay with *Pseudomonas* treated leaves, altered feeding behaviour of the leaffolder larvae, reduction in the larval as well as pupal weight, increase in larval mortality and adult malformation were observed. Besides, these *Pseudomonas* strains also activate the expression of chitinase, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase and phenol accumulation in rice leading to the lignin formation.

Generally in all the experiments, *Pseudomonas* mixture performed better than their individual strain and also the addition of chitin in the talc formulation containing single strain of *Pseudomonas* or strain mixture enhanced the biocontrol activity of *Pseudomonas* strains.

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Introduction

CHAPTER I

INTRODUCTION

Rice, the primary staple food for more than half of the world population is subject to attack by pest and diseases, which often cause considerable loss to the crop. Sheath blight of rice incited by *Rhizoctonia solani* Kuhn and leaffolder (*Cnaphalocrocis medinalis* Guenee) are considered as the most important pests and production constraints in South and South East Asia (Dale, 1994; Ou, 1985). Leaffolder and sheath blight outbreaks have been reported several times in India (Ou, 1985; Yadava *et al.*, 1972), often the sheath blight and leaffolder attack simultaneously leading to complete failure of the crop. The shift from minor to major pest status has been attributed to the adoption of new rice growing practices that accompanied the introduction of high yielding varieties (Litsinger, 1989). The disease cannot be satisfactorily suppressed through host resistance because the inherent level of resistance to sheath blight in rice is low and no cultivar has yet been found to be resistant to the disease. Though effective fungicides and insecticides are available to manage sheath blight and leaffolder, they are not thought of long term solution because of the concerns about exposure risks, health and environment hazards and residue persistence. Moreover, frequent application of pesticides may lead to the development of tolerance in the target organisms. As a result, in recent years the focus has been towards biological control of insect pest and diseases, which is a safe and promising alternative to the use of pesticides. In addition, biocontrol agents are adaptable for mass production and formulation technology and easy delivery system.

The utilization of plant's own defense mechanism is the subject of current interest in the management of pests and diseases. Induced protection of plants against various pests (Karban and Baldwin, 1997) and pathogens (Baker *et al.*, 1997) by biotic and

abiotic inducers has been reported in many crops. The classical inducers include pathogens (Dalisay and Kuc, 1995), plant growth promoting rhizobacteria (PGPR) (Wei *et al.*, 1996; Liu *et al.*, 1995 a; b; c; Leeman *et al.*, 1995), chemicals (Lawton *et al.*, 1994; Ward *et al.*, 1991) and plant products (Singh *et al.*, 1990).

Since the soil-borne *R. solani* and leafhopper cause damage to the aerial parts of the rice plant, the ideal biocontrol agent for these pests therefore should be able to survive in both rhizosphere and phyllosphere. Among the various biocontrol agents, the strains of Plant Growth Promoting Rhizobacteria (PGPR) are known to survive both in rhizosphere and phyllosphere (Krishnamurthy and Gnanamanickam, 1998). PGPR are root colonizers belonging to different genera and most of the reported strains are *Pseudomonas* spp. Recent investigations on mechanisms of biological control by plant growth promoting fluorescent pseudomonads revealed that several strains protect the plants from various pests and pathogens by activating defense genes encoding chitinase, peroxidase and enzymes involved in the synthesis of phytoalexins (M'Piga *et al.*, 1997; Maurhofer *et al.*, 1994) and formation of protective biopolymers, eg. lignin, callose and hydroxy-rich glycoprotein (Hammerschmidt and Kuc, 1982).

Early and increased expression of these genes can induce systemic resistance (ISR) in plants against several pests and diseases. Induced systemic resistance is defined as "any change in plant quality systemically following induction with an inducing agent to a single part of the plant that results in a negative effect on pathogen or herbivore preference for performance on the plant" (Karban and Baldwin, 1997; Kloepper *et al.*, 1992a; Karban and Myers, 1989). Several *Pseudomonas* strains have been reported to induce ISR against many fungal, bacterial, viral and insect pests (Chen *et al.*, 2000; Vidhyasekaran *et al.*, 1999; 1997; Qingwen *et al.*, 1998; Krishnamurthy and Gnanamanickam, 1998; Zehnder *et al.*, 1997; Rapauch *et al.*, 1996; Liu *et al.*, 1995a; b; c; Maurhofer *et al.*, 1994; 1998; Kloepper *et al.*, 1993; Bong and Sikorowski, 1991). The biocontrol efficacy and PGPR activity of *Pseudomonas* spp. were further increased by

mixing two or more strains (Singh *et al.*, 1999; Raupach and Kloepper, 1998; Pierson and Weller, 1994) or mixing with other bacterial or fungal antagonists (Leibinger *et al.*, 1997; Duffy *et al.*, 1996; Janisiewicz, 1996) or mixing with chitin or other substances (Benhamou *et al.*, 1998; El-Ghaouth *et al.*, 1994; Davis *et al.*, 1992). 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, 1999a; Nandakumar, 1998; Wei *et al.*, 1991; 1996). However, the reports on the use of PGPR for induced resistance against arthropod pests are limited (Zehnder *et al.*, 1997).

When plants are treated with PGPR, they are often changed physiologically and biochemically. These changes make the plants less suitable to subsequent attack by herbivores and pathogens. In some of these cases, it appears that plants are benefited from induced responses by reducing subsequent pathogen and pest load (termed induced defense), although it is often difficult to show these benefits by PGPR experimentally. Induced resistance is frequently viewed as an alternative tactic to constitutive resistance and to reduce defense costs by producing them only when needed. Understanding of the relationship between constitutive and induced resistance will be important in defining how best to use induced resistance in agriculture. To use induced responses by PGPR as an effective pest management tool, we must evaluate the effects of induced responses on plant performance and yield under agricultural settings.

Hence in the present study, a talc-based powder formulation of *Pseudomonas* either as single strain or mixtures of strains containing chitin was developed and tested against sheath blight and leafhopper incidence under greenhouse as well as field conditions.

In view of the above background, study was under taken with the following objectives:

- a) Development of talc-based bioformulation containing individual *Pseudomonas* strains and its mixture (with and without chitin) and testing its efficacy against sheath blight disease and leaffolder under glasshouse and field conditions.
- b) Analyzing the induced pattern of defense enzymes in *P. fluorescens* treated plants (with and without inoculation of *R. solani* and leaffolder).
- c) Studying the effect of *Pseudomonas* treatment on different growth stages of leaffolder.
- d) Purification of induced chitinase from *Pseudomonas* treated rice plant and testing its antifungal activity against *R. solani*.

Review of Literature

CHAPTER II

REVIEW OF LITERATURE

2.1. Importance of the pest and disease

Sheath blight, caused by the fungus *Rhizoctonia solani* was a minor rice disease in India until the introduction and wide cultivation of very susceptible, semidwarf varieties. Now this disease is one of the most important and common, that is considered as major production constraint of rice in India and in most rice growing countries of World. It is an economically important disease of rice, being second only to rice blast among the fungal diseases of rice world wide (Ou, 1985; Marshal and Rush, 1980). The disease eventually causes an yield loss of US \$ 72 million in South and South East Asia viz., Japan, Vietnam, South Korea, Taiwan, China, US and India (Herdt, 1991; Ou, 1985). In South India alone, it causes US \$ 15 million yield loss (Ramasamy *et al.*, 1996). In India, this disease occurs in Andhra Pradesh, Assam, Jammu and Kashmir, Orissa, Tamil Nadu and West Bengal (Premalatha Dath, 1990).

The lepidopteran insect viz., leaffolder causes considerable damage to rice crop assuming greater economic importance among the various pests of rice. Leaffolder was once considered as a minor pest of rice but it has become increasingly abundant since intensification of rice cultivation (Chang *et al.*, 1980; Balasubramanian *et al.*, 1973; Dorge *et al.*, 1972). Khan *et al.* (1988) reported the serious menace of *C. medinalis* in tropical and subtropical Asia in last decade. The infestation of leaffolder was also heavy in China, Japan and Korea and in some parts of India, Sri Lanka, Bangladesh, Nepal and Malaysia as observed by Khan *et al.* (1988). The flag-leaf damage ranged from 5 to 85 per cent. They have also observed that 10 per cent increase in damage on leaves reduced the yield by 0.15g/tiller. Feeding on flag leaves by *C. medinalis* was found to be the cause for the maximum yield loss (Murugesan and Chelliah, 1983). Srivastava (1989) indicated by correlation studies that greater the level of infestation, shorter was the

panicle length and lighter, panicle weight. Studies conducted at CRRI, Cuttack during 1982 revealed the prominent occurrence of *C. medinalis* during Kharif and Rabi seasons.

2.2. Symptomatology

Symptoms of the disease usually appear when plants are in the late-tillering or early internode elongation growth stages. Lodging may occur in severely diseased plants, particularly in taller varieties. The natural infection of the sheath blight disease occurs at the seedling, tillering and booting stages of rice. The symptom of the disease includes greenish-grey, elliptical or oval-shaped spots with yellow margin mostly found on leaf sheaths and leaf blades except roots (Ou, 1985; Gangopadhyay and Chakrabarti, 1982). Infection usually starts near the water line of rice plants in paddy fields. Lesion develops upward to the upper leaf sheaths and leaf blades. The centre of lesion becomes greyish white with brown margin, later several spots coalesce and show blight symptoms (Gangopadhyay and Chakrabarti, 1982). Thus entire plant often gets killed under severe cases (Rush and Lindberg, 1984). The symptom of the disease suggests the possible involvement of phytotoxin in lesion development (Vidhyasekaran *et al.*, 1997a; Sriram *et al.*, 1997).

With regard to the leaffolder damage, adults in general are harmless but the larvae are destructive. They fold the leaves longitudinally prior to feeding and feed by scrapping the green mesophyll tissues of rice leaf with in the fold leading to a linear pale white stripe damage (Khan *et al.*, 1988). Rice leaffolder infestation occurs mostly in the vegetative phase of the crop (Fabeller *et al.*, 1994). Jong, (1992) reported that folding of leaves by *C. medinalis* resulted in a significant reduction of net photosynthetic rate.

2.3. Pathogen

The fungus, *R. solani* Kuhn (Perfect stage: *Thanatephorus cucumeris* Frank) is the causative agent of the sheath blight disease. The fungus produces a large number of globose sclerotia which are initially white and later turn to brown or purplish brown. The pathogen survives as sclerotia on the diseased plant debris and non-host plants which

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serve as a primary source of inoculum. The fungus produces the phytotoxin, which is responsible for the symptom on the rice plant (Vidhyasekaran *et al.*, 1997a; Sriram *et al.*, 1997)

2.4. Insect

Adult female of *C. medinalis* lives for 8 to 10 days. Eggs are normally deposited on the upper surface of leaves in between the veins of leaves. The eggs are occasionally laid on the lower surface. Eggs are laid singly or in-groups and hatch within 3 to 4 days. The first instar larvae move towards the leaf tips in search of a suitable leaf to make fold. Within the leaf fold normally only one larva is found. Both the ends of the fold are found to be open. The larvae are able to scrape the green matter between veins leading to a white thin papery surface (Yadava *et al.*, 1972). Grown up larvae turned orange in colour and start spinning thin silken cocoon with an exit hole before entering pupation. The pupal period lasts for 6 to 10 days and the most of the moths emerge during evening hours. Moths are normally found to mate during night time and copulation lasts for five minutes. The longevity of the female and male is 8 to 10 and 2 to 3 days respectively (Nanda and Bisoi, 1990).

2.5. *P. fluorescens* as potential biocontrol agent

Bacteria are by far the most common type of soil microorganisms, possibly because they can grow rapidly and have the ability to utilize a wide range of substances as either carbon or nitrogen sources. The bacteria that provide some benefit to plants are found near, on or even within the roots of plants (Kloepper *et al.*, 1980; Van peer and Schippers, 1988). Among the many potential bacterial antagonists associated with the plant roots, the fluorescent pseudomonads have received prominent attention due to their abundance in plant rhizosphere and their ability to colonize roots of a wide range of crop plants. Fluorescent pseudomonads have been chosen because these bacteria are most widely studied for their potential to protect plants against diseases and to promote plant growth (Cook *et al.*, 1988). In the past three decades, numerous strains of fluorescent pseudomonads have been isolated from the soil and plant roots by several workers

(Rabindran and Vidhyasekaran, 1996; Rosales *et al.*, 1993; Mew and Rosales, 1986; Austin *et al.*, 1977).

2.5.1. Biocontrol properties

Cook and Rovira (1976) reported that the take-all disease of wheat caused by *Guamannomyces graminis* var *tritici* was suppressed by certain strains of fluorescent pseudomonads that were isolated from the natural soil. Treatment with certain strains of fluorescent pseudomonads showed enhanced resistance in sugarbeet to the fungus *Pythium ultimum* which causes pre and post emergence damping-off (Osburn *et al.*, 1983) and seedling infection in cotton (Gutterson *et al.*, 1986).

Suppression of *S. rolfii* in groundnut and *R. solani* in rice by the strains of fluorescent pseudomonads under *in vitro* and *in vivo* conditions was reported (Vasanthadevi *et al.*, 1989; Ganesan and Gnanamanickam, 1987). Soil application of fluorescent pseudomonads strain E113 isolated from tulip roots at a concentration of 10^8 cfu/g of dry soil suppressed the root rot pathogen, *P. ultimum* and was considered to be more effective than applying the bacterium to the bulbs (Weststeijn, 1990). Hagedorn *et al.* (1993) achieved improved seedling stand in cotton through furrow application of *P. fluorescens* at the rate of 14.1 ml/m. The improved seedling stand was due to the suppression of seedling diseases by antagonistic fluorescent pseudomonads.

Seed treatment with *P. fluorescens* strain WCS 417 has protected radish through induction of systemic resistance not only against the fungal root pathogen *F. oxysporum* f. sp. *raphani*, but also against the avirulent bacterial leaf pathogen *P. syringae* pv. *tomato* and fungal leaf pathogens *Alternaria brassicola* and *F. oxysporum* (Hoffland *et al.*, 1996). This implies that same PGPR strain can induce resistance against multiple pathogens in the same crop. ISR by *P. putida* strain 89 B - 27 and *S. marcescens* strain 90-166 against anthracnose of cucumber was established by Wei *et al.* (1991). Later studies showed that the same PGPR strains induced systemic protection against angular leaf spot caused by *P. syringae* pv. *lachrymans* (Liu *et al.*, 1993a), *Fusarium* wilt incited by *F. oxysporum* f.sp. *cucumerinum* (Liu *et al.*, 1993b) and cucurbit wilt caused by *Erwinia tracheiphila*

(Kloepper *et al.*, 1993). Seed treatment of *S. marcescens* strain 90-166 has shown ISR in cucumber against anthracnose, cucumber mosaic virus, bacterial angular leaf spot and cucurbit wilt diseases (Liu *et al* 1995a; 1995b; Kloepper *et al.*, 1993).

P. fluorescens strain Pfl induces resistance against different pathogens viz., *Rhizoctonia solani* (Nandakumar, 1998), *Colletotrichum falcatum* in sugarcane (Viswanathan, 1999). The broad spectrum of PGPR-mediated ISR is more rewarding than narrow spectrum of disease protection.

PGPR-mediated ISR against insects are also available but are restricted to very few crops. Generally, fluorescent pseudomonads influence the preference of the plant, growth and development of insects at all stages of growth (egg, larva, pupa and adult). *Pseudomonas maltophilia* affects the growth of larval stage of *Helicoverpa zea*, the corn earworm, leading to more than 60 per cent reduction in adult emergence. Pupae and adults emerging from the bacteria-infected larvae remained smaller (Bong and Sikorowski, 1991). Similarly, the relative growth rate, consumption rate and digestibility of feed by larvae of *Helicoverpa armigera* have been affected when they have fed on cotton plant treated with *Pseudomonas gladioli* due to increased polyphenol and terpenoid content in treated plants (Qingwen *et al.*, 1998). As fluorescent pseudomonads are effective rhizosphere colonizers and are endophytic in nature in the plant system, attempts have been made to transfer the insecticidal crystal protein from *Bacillus thuringiensis* to *P. fluorescens*. Thus genetically engineered *P. fluorescens* has been found to be effective against lepidopteran insect pest. Transgenic *P. cepacia* strain 526 with the crystal protein gene has consistently shown insecticidal activity against tobacco hornworm (Stock *et al.*, 1990). Induction of systemic resistance by PGPR against various diseases and pests was considered as the most desirable approach in crop protection.

2.5.2. Mode of action

The early studies with fluorescent pseudomonads demonstrated control of soil-borne pathogens through various modes of action of biological control. Disease suppression by fluorescent pseudomonads depends mainly on competition for nutrients and space (Elad and Baker, 1985), its ability to colonize rhizosphere (Scher *et al.*, 1988), to produce antibiotics *viz.* pyrrolnitrin, pyoverdine, 2,4-diacetylphloroglucinol (Gardener *et al.*, 2000; Hammer *et al.*, 1995; Maurhofer *et al.*, 1995; Pierson and Thomashow, 1992), siderophore (fluorescent yellow green pigment) *viz.* pseudobactin which chelates the iron thereby limiting the availability of iron necessary for the growth of pathogens (Loper, 1988), lytic enzymes such as chitinases and β -1,3-glucanases which degrade chitin (major cell wall component of higher fungi, major components of exoskeleton and peritrophic membrane of insect and nematodes) and glucan, respectively, (Velazhahan *et al.*, 1999; Broadway *et al.*, 1998; Sikora, 1992; Frindlender *et al.*, 1991). In addition, production of hydrogen cyanide degrade the toxin produced by the pathogen (Duffy and Defago, 1997; Borowitz *et al.*, 1992; Voisard *et al.*, 1989) and salicylic acid (Maurhofer *et al.*, 1998; De Meyer and Hofte, 1997) have also been involved in the suppression of soil-borne pathogens.

More recently, three laboratories independently demonstrated that certain PGPR strains protected plants through mechanisms associated with ISR against pathogens that cause foliar disease symptoms (Alstrom, 1991; Van Peer *et al.*, 1991; Wei *et al.*, 1991). Subsequent research at Auburn University has demonstrated PGPR-ISR against various fungal and bacterial pathogens of cucumber (Liu *et al.*, 1995 a; b). Improved efficacy and enhanced biological control consistency against multiple pathogens can be achieved by applying a combination of PGPR strains (Raupach and Kloepper, 1998). Interestingly, induction of systemic resistance by prior application of fluorescent pseudomonads protect the crop against pests and diseases by strengthening the cell-wall structure and causing biochemical and physiological changes in the plant system (Chen *et al.*, 2000; Kloepper *et al.*, 1997; Zehnder *et al.*, 1997a; Hoffland *et al.*, 1996; Wei *et al.*, 1996).

Microbial control of insects, *i.e.*, the use of microorganisms or their byproducts to suppress pest population or damage has a long history (Steinhaus, 1975). The endophytes provide biological control of insects, but they do not fall into any of the earlier categories of the natural enemies of the insects referred to as “predators, parasites and pathogens”. (De Bach, 1964). Zehnder *et al.* (1997b) indicated that PGPR-treated plants of both cultivars contained significantly lower levels of cucurbitacin than nontreated plants. This suggested that a mechanism for PGPR-induced resistance against cucumber beetle might involve a change in the metabolic pathway for cucurbitacin synthesis. Support for this hypothesis comes from previous studies demonstrating that metabolic pathway for cucurbitacin and other plant defense compounds share similar precursors (Balliano *et al.*, 1982).

2.5.3. Formulation and application

Cell suspension of the bacteria has been used for seed treatment, but the use of such suspension is impractical for large-scale field application (Thompson, 1996; Capper and Higgins, 1993; Mew and Rosales, 1986). A powder formulation with a longer shelf life would be beneficial. Different carrier formulation of fluorescent pseudomonads has been developed. The talc based powder formulation containing antagonistic bacterium was effective against chickpea wilt, pigeonpea wilt, rice blast and rice sheath blight (Vidhyasekaran and Muthamilan, 1999; Vidhyasekaran *et al* 1997a; b; Rabindran and Vidhyasekaran, 1996) and sugarcane red rot (Viswanathan and Samiyappan, 1999). The development of the powder formulation of the bacteria with a shelf life of more than 8 months (Vidhyasekaran and Muthamilan, 1999) and an efficient method of application may be highly useful for large scale field application of the product and effective control of the diseases.

Seed treatment followed by root zone application of talc-based antagonistic formulation effectively controlled the chickpea wilt disease in two field trials and increased the yield significantly. Similarly seed treatment with powder formulation of fluorescent *Pseudomonas* at the rate of 4g/kg of seeds followed by soil application with 2.5 kg per hectare of land at 0, 30 and 60 days after sowing showed enhanced resistance

to pigeonpea wilt. The methods of application include seed treatment (Rosales and Mew, 1997; Liu *et al.*, 1995a,b; Van Peer and Schippers, 1988), seedling root dip (Nayar, 1996; Maurhofer *et al.*, 1994), soil application (Vidhyasekaran *et al.*, 1997b; c; Samiyappan, 1988) and foliar application (Chatterjee *et al.*, 1996; Mew and Rosales, 1986). Combination of individual treatments can also be practiced (Nandakumar, 1998; Vidhyasekaran *et al.*, 1997b; Rabindran and Vidhyasekaran, 1996). Both seed and soil application increased the yield compared to seed treatment alone (Vidhyasekaran *et al.*, 1997c).

2.5.4. Effect of PGPR strain mixtures

Mixtures of different PGPR strains have resulted in enhanced biocontrol efficacy against the pathogen. An effective biological control results from mixtures of antagonists rather than from high populations of a single antagonist. Similarly, application of a mixture of introduced biocontrol agents would more closely mimic the natural situation and might broaden the spectrum of biocontrol activity and enhance the efficacy and reliability of control (Duffy and Weller, 1995). Application of a mixture of three PGPR strains viz., *Bacillus pumilus* strain INR 7, *B. subtilis* strain GB03 and *Curtobacterium flaccumfaciens* strain ME1 as seed treatment has resulted in much more intensive plant growth promotion and disease reduction when compared to strains tested singly. This might be due to different mechanisms of action for each PGPR strain (Raupach and Kloepper, 1998). Use of chitinase-producing *Streptomyces* spp. and *Bacillus cereus* isolates used in combination with antibiotic-producing *P. fluorescens* and *Burkholderia* (*Pseudomonas*) *cepacia* isolates have showed a synergistic effect on the suppression of rice sheath blight incited by *R. solani* (Sung and Chung, 1997). Application of mixture of two chitinolytic bacterial strains viz., *Paenibacillus* sp. 300 and *Streptomyces* sp. 385 in the ratio of 1:1 or 4:1 has been found to be effective than when they are applied individually for the control of *Fusarium* wilt of cucumber incited by *F. oxysporum* f. sp. *cucumerinum* (Singh *et al.*, 1999). Similarly, mixtures of *Pseudomonas* spp. have been more suppressive to take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* than treatment using single *Pseudomonas* sp. (Duffy and Weller, 1995; Pierson and

Weller, 1994). Combination of *P. fluorescens* strains viz., Pfl and FP7 has given effective control of rice sheath blight disease when compared to strain applied singly (Nandakumar, 1998).

However in certain cases, mixtures of different strains have no synergistic effect. Further, a mixture that improves efficacy under one set of conditions or on one host may not perform under different conditions and also on different hosts (Schisler *et al.*, 1997). From the economic point of view, a biocontrol product composed of a mixture of strains may be more expensive than a product composed of single strain. While developing mixture of strains, greater emphasis must be given on their efficacy against disease suppression by ISR.

2.5.5. Endophytic nature

Endophytic bacteria colonize an ecological niche similar to that of plant pathogen, especially vascular wilt pathogens, which might favour them as potential antifungal agents. Kloepper *et al.* (1992b) 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 *Pseudomonas syringae* pv *lachrymans* as well as to the fungal pathogens *Fusarium 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).

2.6. Induction of systemic resistance

Induced protection in plants against various pathogens by biotic or abiotic inducers has been reported since 1930s when Chester (1933) used the term “acquired physiological immunity”. Later different authors used different terms to describe the phenomenon of induced resistance such as translocated resistance (Hubert and Helton, 1967) and plant immunization (Tuzun and Kuc, 1991).

Induced systemic resistance (ISR) by definition, refers to protection of the plants systemically by enhancement of plant's defensive capacity against a broad spectrum of pathogens and pests that is acquired after appropriate inducing agent upon infection by a pathogen or pest. Induction of systemic resistance by PGPR against various diseases and pests was considered as the most desirable approach in crop protection. There are major differences in ISR when compared to other mechanisms. Fluorescent pseudomonads appear to be ideal for this purpose because some strains can induce systemic resistance in plants against fungal, bacterial, viral diseases and insects (Van Loon *et al.*, 1998; Zehnder *et al.*, 1997a; Wei *et al.*, 1996, Maurhofer *et al.*, 1994; Alstrom, 1991; Van Peer *et al.*, 1991). First, the action of ISR is based on the defense mechanisms that are activated by inducing agents. Secondly, ISR once expressed activates multiple potential defense mechanisms that include increased activity of chitinases, β -1,3-glucanases and peroxidases (Xue *et al.*, 1998; Dalisay and Kuc, 1995; Maurhofer *et al.*, 1994; Schneider and Ullrich, 1994) and accumulation of antimicrobial low molecular weight substances phytoalexins (Van Peer and Schippers, 1992; Van Peer *et al.*, 1991) and formation of protective biopolymers, eg. lignin, callose and hydroxy proline-rich glycoprotein (Hammerschmidt and Kuc, 1982). Thirdly, an important aspect of ISR is the wide spectrum of pathogens that can be controlled by single inducing agent (Hoffland *et al.*, 1996; Wei *et al.*, 1996; Dean and Kuc, 1985). Thus ISR appears to be the result of several mechanisms which together are effective against wide range of fungal, bacterial and viral pathogens. They fit more logically into the plant pathology concepts of "antagonist". Induced responses and induced resistance have been well documented and have been found in many plant taxa (Karban and Baldwin, 1997). Induced responses to herbivory are "immune-like" responses that reduce the performance or preference of herbivore (or both). Plants have evolved complex and varied defense mechanisms for protection against herbivore and disease. These mechanisms may be constitutive (e.g., active throughout the plant's life) or induced following attack by herbivores or pathogens.

Recent studies have suggested that inducible defenses in plants may have selective advantages over constitutive defenses (Agrawal, 1998 and references therein). Plant

colonization by green peach aphids (*Myzus persicae*) was affected by induction in a similar manner with 30% more aphids on controls compared with induced plants. (Agrawal, 1998). Plant defense mechanisms may be activated systemically throughout the plant following a localized infection or attack (Kessman *et al.*, 1994).

Various "classical" inducers of ISR have been reported, including pathogenic microorganisms like fungi, bacteria and viruses (Chittoor *et al.*, 1997), and insect pests (Karban and Baldwin 1997; Kombrink *et al.*, 1988), synthetic chemicals, and metabolic products of hosts or infectious agents (Liu *et al.*, 1995c), PGPR (Liu *et al.*, 1995a, b; Schneider and Ullrich, 1994), plant products (Singh *et al.*, 1990) and incompatible host pathogen interactions (Reimers *et al.*, 1992). However reports of induced resistance in the field by classical inducing agents have been infrequent. Plant defense mechanisms may be activated systemically throughout the plant following a localized infection or attack (Kessman *et al.*, 1994).

2.6.1 Mechanism of induced systemic resistance

The fluorescent pseudomonads bring about ISR through fortifying the physical and mechanical strength of the cell wall as well as changing the physiological and biochemical reaction of the host leading to the synthesis of defense chemicals against the challenge pathogen.

2.6.1.1. Biochemical and Physiological changes in the PGPR treated plants

The PGPR strains belonging to *Pseudomonas* spp. induce several defense genes encoding pathogenesis-related (PR) proteins viz., chitinase, β -1,3-glucanase, PR1 proteins, peroxidase, chalcone synthase, phenylalanine ammonia-lyase (PAL) and induce accumulation of phenolics, callose, lignin and phytoalexins. Hynes and Lazarovits (1989) found changed protein profile in bean leaves after seed treatment with PGPR strains. Zdor and Anderson (1992) reported that inoculation of *P. aureofaciens* in bean plants induced more of 57 kDa protein and *P. putida* and *P. tolaasi* induced more of 38 kDa protein in leaves.

2.6.1.1 PR-proteins

2.6.1.1.1 Chitinases

Chitinases (EC 3.2.1.14) are PR-proteins which hydrolyze chitin, a major cell wall component (3-10%) of higher fungi. Chitinases 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 of 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). Many workers have purified the chitinases and proved its antifungal activity against fungal pathogens (Lawrence *et al.*, 1996; Ji and Kuc, 1996). Chitinases degrade the fungal cell wall chitin and inhibited the fungal growth at hyphal tip (Velazhahan *et al.*, 2000; Schlumbaum *et al.*, 1986; Boller *et al.*, 1983). It was demonstrated that chitinase accumulates around the fungal cell wall *in planta* (Benhamou *et al.*, 1994; Wubben *et al.*, 1992). Some chitinases also display lysozymal activity (Majeau *et al.*, 1990). The potential of chitinases for use in biocontrol has stimulated to isolate the gene encoding these enzymes and cloning them into plants. It has been now demonstrated that enhanced chitinases in plants can indeed reduce the damage caused by pathogens (Brogie *et al.*, 1991).

In recent years, several biocontrol agents like PGPR have been shown to induce systemic resistance in plants. Accumulation of chitinases and peroxidases with the onset of ISR by PGPR has been observed in some plants. Enhanced accumulation of chitinase in tobacco and bean leaves was observed in response to application to *Pseudomonas* spp. inoculation 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 *Pseudomonas syringae* pv. *tabaci* (Schneider and Ullrich, 1994). Nandakumar (1998) and Nayar (1996) found early and high induction of chitinase in *P. fluorescens* Pfl treated rice plants. Such

enhanced induction of chitinase offered protection against *Rhizoctonia solani* in rice. Recently, 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 31, 25 and 23 kDa in rice.

Exogenous application of certain chemicals and increasing the endogenous level of chemical composition has been shown to trigger systemic resistance. Salicylic acid (SA), ethylene, 2,6 iso-nicotinic acid (INA) are the well-known inducers of systemic resistance in plants (Dann *et al.*, 1996; Lawton *et al.*, 1994; Uknes *et al.*, 1992). Salicylic acid application induced gene families that encode PR-proteins viz. acidic extracellular β -1,3glucanases and acidic extra cellular chitinases in tobacco (Ward *et al.*, 1991) and mRNA's encoding acidic and basic chitinases (Lawton *et al.*, 1994). Treatment with INA in bean caused changes in chitinase and β -1,3-glucanase activity after inoculation with *C. lindemuthianum* and also responsible for changes in free and bound salicylic acid (Dann *et al.*, 1996). Transcription of chitinase genes was strongly stimulated by exogenously applied ethylene in bean leaves (Boller *et al.*, 1983). Increased chitinase synthesis in plants after ethylene treatment was reported by several workers (Broglie *et al.*, 1986; Boller *et al.*, 1983). Broglie *et al.* (1991) demonstrated that transgenic tobacco and canola plants constitutively expressing an ethylene inducible bean chitinase showed enhanced resistance to the chitinaceous fungus *R. solani* but not to the non chitinaceous fungus, *Pythium aphanidermatum*.

The bean chitinase accumulated at level upto 2 % of total soluble protein, it provided no protective effect on the tomato moth larvae (Gatehouse *et al.*, 1997). However, small but statistically significant decrease in the growth and fecundity of the peach-potato aphid, *Myzus persicae* on transgenic potato plants expressing multiple genes of chitinase. The transgenic plants expressing the insect chitinase appear to be more resistant to feeding damage by tobacco hornworm and tobacco budworm (Ding *et al.*, 1997; Ding, 1995).

However, the role of endogenous chitinase from various sources in mediating plant resistance to insects is less well understood.

2.6.1.1.1.2. 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, 1997). Bradley *et al.* (1992) reported the increased peroxidase (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 precursor into plant cell wall, thus constructing a physical barrier that could prevent pathogen penetration of cell walls or 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 are the best inducers of plant chitinase and peroxidases, which are the most important components of the induced systemic resistance (Nandakumar *et al.*, 2000). Two peroxidase isoforms have been induced in the PGPR-treated plants inoculated with the sheath blight pathogen, *R. solani* (Nandakumar *et al.*, 2000). These enzymes are also part of the response of plants to pathogens (Hammerschmidt and Kuc, 1995) and they may decrease the quality of these plants as host for insects (Duffey and Stout, 1996). In common bean seedlings, exposure to controlled wind-induced stress reduced fungal anthracnose (*C. lindemuthianum*) lesion development and two-spotted spider mite reproduction (Cipollini, 1997). Although peroxidase alone has little effect on insect (Dowd and Vega, 1996; Rahbe and Febvay, 1993; Felton and Duffey, 1991), there was some evidence that peroxidases can contribute to insect resistance. Oxidation products of several corn allelochemicals produced by peroxidases were more toxic than precursor when produced in sucrose solution fed to the maize leaf-hopper *Dalbulus maidis* (Dowd and Vega, 1996). An inbreed of maize with kernel resistance to *Aspergillus flavus* (Scott and Zummo, 1988) and the dusky sap beetle, *Carpophilus lugubris* (Dowd, 1994) compared to

a susceptible inbred shows a rapid browning response when damaged. Subsequent analysis indicated that the resistant variety had several additional peroxidase isoenzymes compared to susceptible inbred (Dowd, 1994). Maize callus derived from the resistant inbred that was brown was more resistant to feeding by *H. zea* (Dowd and Norton, 1995) and the brown material was found to have additional peroxidase isoenzymes. In a field test, transgenic tomato expressing the tobacco anionic peroxidase showed significantly lower number of adult lady beetles compared to the wild type (Dowd and Lagrimini, 1996).

2.6.1.1.2 Polyphenol oxidase (PPO)

PPO usually accumulates 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. The increased activation of PPO could be detected in the cucumber leaf in the vicinity of lesions caused by some foliar pathogens. Moreover, 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 plants (Viswanathan, 1999).

Thaler *et al.* (1999) reported that the octadecanoid pathway was induced by measuring more PPO activity (a putative defense) in the induced tomato plants than the control plants four days after the plants were sprayed with jasmonic acid. The octadecanoid pathway, usually associated with feeding by chewing insect or similar physical trauma, involves jasmonic acid as an intermediate signal and culminates in the production of proteins such as PPO and proteinase inhibitors (Schaller and Ryan, 1995). Application of jasmonic acid results in induction of proteinase inhibitors and PPO resulting in decrease in the preference, performance and abundance of many common herbivores in the field, including *Frankliniella occidentalis* (thrips), *Spodoptera exigua* and *Trichoplusia ni*

(noctuid caterpillars), *Epitrix hirtipennis* (flea beetles) and *Myzus persicae* (aphids) (Thaler *et al.*, 1996).

2.6.1.1.3. Phenylalanine ammonia-lyase (PAL)

The activation of the phenylpropanoid pathway in plants by environmental stimuli is one of the most universal biochemical stress responses known. PAL catalyzes the deamination of L-phenylalanine to 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 peroxidase and the accumulation of such phenolics as 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, which express the elicitor-inducible PAL have been developed. The transgenic rice plants expressing PAL ZB8 showed systemic resistance against rice pathogens (Lamb *et al.*, 1997). Seed treatment and seedling root dipping with PGPR induced early and enhanced levels of PAL in rice plants (Nayar, 1996). Plants treated with *Pseudomonas* strains had initially higher levels of PAL, but these levels were lower in control (Chen *et al.*, 2000).

2.6.1.2. Strengthening the cell wall structures

The rapid strengthening reaction at sites of fungal and insect entry delays the infection process and allows sufficient time for the host to build up other defense reactions. Seed treatment of 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 the walls of these cells 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 seen 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.*, 1996), 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 *et al.*, 1998).

2.6.2. Bacterial traits involved in ISR

Limited information is available on the bacterial traits involved in PGPR-mediated ISR. Van Peer and Schippers (1992) obtained evidence that lipopolysaccharides (LPS) extracted from outer membrane of *P. fluorescens* WCS 417 induced systemic resistance in carnation to *F. oxysporum* f. sp. *dianthi*. Maurhofer *et al.* (1994) showed the involvement of the pyoverdine siderophore of *P. fluorescens* CHAO in the induction of disease resistance against TNV in tobacco. Though cyanide production was earlier correlated with ISR (Wei *et al.* 1991; Voisard *et al.*, 1989) found no evidence for the role of cyanide in ISR against *C. orbiculare* on cucumber. Leeman *et al.* (1995) found that the O-antigenic side chain of LPS, present on the outer membranes of strains of WCS 374 and WCS 417 of *P. fluorescens* may be involved in ISR in radish. Van Wees *et al.* (1997) observed that rhizosphere pseudomonads are differentially active in eliciting ISR in related plant species. Recently, De Meyer and Hofte (1997) reported that salicylic acid and pyochelin production by *P. aeruginosa* 7NSK2 was essential for the induction of resistance to *Botrytis cinerea* in bean. The transcriptional activity of salicylic acid and pyochelin biosynthetic genes was detected during *P. aeruginosa* 7NSK2 colonization in the bean and iron nutritional state at inoculation also influenced the transcriptional activity of the genes.

2.6.3. Durability of ISR

Resistance mechanisms attain maximum effectiveness at four to five days after the application of an inducing agent, but the level of persistence of resistance generally decreases over time. Studies on durability of ISR is useful to determine the number of applications of PGPR and time interval for application needed to maintain the resistance level in the crop plants (Dalisay and Kuc, 1995). When rice seeds were treated with *P. fluorescens* Pfl strain, resistance in foliage was observed upto 45 days after sowing. The ISR was observed three to four days after foliar spray and the resistance persisted upto 15 days in rice leaves (Vidhyasekaran *et al.*, 1999). Foliar sprays of *P. fluorescens* formulations should be given at every 15 days intervals for managing rice foliar diseases. Parallel experiments conducted by Nayar, (1996) indicated that induction of defense mechanisms using *P. fluorescens* persisted up to 60 days by seed treatment, 30 days by root dipping and 15 days by foliar spray. In cucumber, PGPR-mediated ISR resulted in protection of cucumber from anthracnose disease for five weeks from the time of sowing. But the consistency of ISR over time varies with PGPR strains (Liu *et al.*, 1995c). In sugarcane, induction of resistance by PGPR persists for 90 days of crop growth (Viswanathan, 1999). Generally, the durability of resistance by PGPR differs from crop to crop and also due to different bacterial strains.

2.6.4. PGPR mediated ISR under field conditions

Efficacy of PGPR strains against soil borne plant pathogens has been proved under field conditions (Vidhyasekaran *et al.*, 1997c; Rabindran and Vidhyasekaran, 1996; Vidhyasekaran and Muthamilan, 1995). But only in few cases, PGPR mediated ISR was demonstrated in the field. In four consecutive years, seed treatment with resistance inducing *P. fluorescens* strain WCS374 reduced *Fusarium* wilt of radish in a naturally infested green house by upto 50% (Leeman *et al.*, 1995). Similarly, 4 PGPR strains applied as seed treatments followed by a soil drench at-transplanting suppressed angular leaf spot and anthracnose of cucumber in three field trials over a period of two years (Wei *et al.*, 1996). In all the three trials, most of the PGPR strains promoted early season growth

and enhanced yield. Under similar conditions, naturally occurring bacterial wilt, caused by *Erwinia tracheiphila* was also significantly reduced (Kloepper *et al.*, 1993).

2.6.4.1 Natural enemies population on the induced resistant plant

Plants have evolved to use parasites and predators as a defense against phytophagous insects, phytochemicals of the host plants acting as long range cues for attracting natural enemies. The natural enemies associated with leaffolder vary with different ecosystems and regions. These natural enemies are also essential for maintaining insect population at low level. It is very difficult to develop a management programme unless natural enemies are identified and their roles are understood (Shepard and Ooi, 1991). Herbivore-infested tomato plants release increased amounts of volatiles and attracts natural enemies of the herbivores (Dicke *et al.*, 1998), as do other plants (De Moraes *et al.*, 1998; Hopke *et al.*, 1994). *Spodoptera exigua* larvae feeding on induced tomato plants have reduced growth rates compared to larvae feeding on control plants and are primarily parasitized in the late first and second instar (Thaler *et al.*, 1996). Occurrence of the *Hyposoter exigua* (endoparasitic wasp of economically important pest, *Spodoptera exigua*) pupae was twice on the induced plants due to induction of plant-herbivore-natural enemy interaction in the field. In cotton and tobacco field trials using female wasps (*Cardiochiles nigriceps*), the ratio of landings on host (tobacco budworm) damaged versus undamaged plants was high: approximately 95% to 5%, respectively in systemic or whole-plant volatile emissions (De Moraes *et al.*, 1998). In tobacco, higher concentrations of the defense-signaling molecule jasmonic acid result from herbivore damage by hornworm caterpillars than from mechanical damage designed to mimic herbivory (McCloud and Baldwin, 1998).

2.7. 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 *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 are also found as potential elicitors of plant defense reactions (Benhamou and Theriault, 1992; Leuba and Stossel, 1986). Recently, Benhamou *et al.* (1998) found induction of resistance against *Fusarium* wilt by combination of chitosan with an endophytic bacterium *Bacillus pumilus* strain SE 34 in tomato.

Chitin amendment drastically reduced the number of both stubby root nematodes (*Trichodorus* spp. and *Collembola* (*Protaphorura* spp.), millipedes (*Brachydesmus* spp.) and wire worm (*Agriotes* spp.) as reported by Ellis *et al.*, (1998).

2.8. Bacterial chitinases

Chitinases are widely distributed in nature and play an important role in the hydrolysis of β -1, 4-linkages of GlcNAc polymer chitin, a structural polysaccharide present in different organisms mainly arthropods and fungi (Cabib, 1987). Chitinases are produced by a number of bacteria and fungi living in chitin containing habitats such as soil, sediments and marine environments. Among the bacteria, *Pseudomonas* spp., *Bacillus* spp. and *Streptomyces* spp. are chitinolytic (Gooday, 1990). Chitinolytic activity of *Bacillus amyloliquefaciens* (El-Aassar *et al.*, 1992), *Streptomyces thermoviolaceus*, *Serratia marcescens* and *B. circulans* (Tsujibo *et al.*, 1992), *Streptomyces viridificans* and *Serratia marcescens* (Gupta *et al.*, 1995) and *Flavobacterium* sp (Frandsberg and Schnurer, 1998) were reported. Frandsberg and Schnurer (1998) reported that chitinolytic bacteria from airtight stored cereal grain had strong inhibitory effect on moulds like *Aspergillus* spp. and *Penicillium* spp. They also found that addition of 0.15% chitin in tripticase-glucose extract medium recorded higher chitinase activity and more mycelial growth inhibition. *Serratia marcescens*, a soil bacterium possesses good chitinolytic activity, suggesting that this was a significant factor in the bacterial antifungal activities against *S. rolfii* (Ordentlich *et al.*, 1988). Chitinase and laminarinase produced by *Pseudomonas stutzeri* strain VPL-1 reduced the growth of *Fusarium solani* (Lim *et al.*, 1991).

2.9. Direct growth promotion

During 1970's specific rhizosphere bacteria applied to seeds were reported to colonize roots and promote plant growth (Kloepper and Schroth, 1978) and were later termed as plant growth promoting rhizobacteria (PGPR). PGPR are plant root colonizers belonging to different genera and species. Certain PGPR strains enter into the plant system and colonize root cortex region and function as endophytes. Most of the reported PGPR strains are from *Pseudomonas* and *Bacillus* spp. Their continual association with the crop roots makes PGPR an excellent candidate for delivering beneficial effect to the root zone.

The studies of mechanism indicated that PGPR promote plant growth directly by the 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 not explained clearly. Barea *et al.* (1976) observed 50 phosphate dissolving bacteria for IAA, gibberellin and cytokinin production. Among them, 17 isolates of *Pseudomonas* produced auxin or cytokinin and gibberellin.

Seed treatment with PGPR causes increased yield and growth in potato tuber 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% respectively, when the bacteria were applied to the seed (Sakthivel *et al.*, 1986).

The plants grown from the seeds treated with both fluorescent and non-fluorescent bacterial isolates grew faster and were taller and greener than untreated control. 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.* (1991a). 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 seedling in sugarbeet when compared to seedlings from untreated seeds (Williams and Asher, 1996).

Materials and Methods

CHAPTER III

MATERIALS AND METHODS

3.1. Seed material

Rice 'IR 50', a cultivar highly susceptible to sheath blight (*R. solani*) and leaf folder (*C. medinalis*) was used in all experiments for evaluating the responses of bacterized rice plants to infection with sheath blight and leaf folder. The seed material was obtained from Paddy Breeding Station, TNAU, Coimbatore.

3.2. Source of antagonistic strains and growth conditions

3.2.1. Isolation of fluorescent pseudomonads from rhizosphere soil of paddy

One g of rhizosphere soil adhering to the root surface was collected and transferred to a 250 ml conical flask containing 100 ml of sterile water. After thorough shaking for 15 min in shaker, different dilutions were prepared. One ml of each 10^{-5} and 10^{-6} dilutions was pipetted out and poured into the sterile Petri dishes. King's B (KB) medium (King *et al.*, 1954) was poured, rotated and incubated at room temperature ($28 \pm 2^\circ\text{C}$) for 24 h.

P. fluorescens isolates PF1 and FP7 were also obtained from Dept. of Plant Pathology, TNAU, which was isolated from the rhizosphere of rice plants in the field. Strains PF1 and FP7 were kept at -80°C in 44 % glycerol and cells from stocks were first grown in King's broth. Inoculum was produced by transferring two loopfuls from the culture to 100 ml of KB broth in a 250 ml Erlenmeyer flask and incubating at room temperature ($28 \pm 2^\circ\text{C}$) on a shaker at 150 rpm for 48 h. These strains were subcultured once in a month and maintained until the end of experiment in KB slants.

3.2.2. Preparation of bacterial suspension inoculum

The bacteria were grown on KB broth with constant shaking at 150 rpm for 48 h at room temperature ($28 \pm 2^\circ\text{C}$). The bacterial cells were harvested by centrifugation at

6000 rpm for 15 min and bacterial cells were resuspended in phosphate buffer (0.01 M, pH 7.0). The concentration was adjusted using a spectrophotometer to approximately 10^8 cfu/ml ($OD_{595}=0.3$) and used as bacterial inoculum (Thompson, 1996).

3.2.3. Preparation of talc-based formulation of PGPR strains

3.2.3.1. Talc-based formulation for individual strain

A loopful of bacterium was inoculated into the KB broth and incubated in a rotary shaker at 150 rpm for 48 h at room temperature ($28\pm 2^\circ\text{C}$). After 48 h of incubation, the broth containing 9×10^8 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 10 g (adhesive) were mixed under sterile conditions, following the method described by Vidhyasekaran and Muthamilan (1995). After shade drying for over night it was packed in polypropylene bag and sealed. At the time of application, the population of bacteria in talc formulation was 2.5 to 3×10^8 cfu/g.

3.2.3.2. Talc-based formulation for strain mixtures

The bacterial strains were grown separately and two strains which are going to make up the mixture were added equally (v/v) and finally mixed with talc powder, CaCO_3 and CMC as mentioned in 3.2.3.1.

3.2.3.3. Chitin amendments with talc-based formulations

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 the pH became neutral. The concentration was adjusted to 10 mg per ml and added to KBB (1%, v/v). The broth containing 9×10^8 cfu/ml after 48 h of incubation was used for the preparation of talc-based formulation as described above.

3.3. Mass rearing of leaffolder

Live moths were collected from the Paddy Breeding Station, Coimbatore and cultured in the greenhouse for further studies. The leaffolder larvae were released into oviposition cages (Plate 1) of 45x45x60 cm size carefully using the camel brush on to the leaves of the IR50 rice plants. Ten per cent honey solution was provided in the cages as food to maintain the stock culture following the methods by Waldbauer and Marciano (1979); Fujiyoshi *et al.* (1980) and Godase and Dumbre (1982).

3.4. Plant growth promotion study

3.4.1. Effect of *P. fluorescens* and its formulation on seed germination and seedling vigour under laboratory conditions

The rice seeds were surface sterilized with 1% sodium hypochlorite and seeds were treated with the pure culture of *P. fluorescens* with a concentration of 3×10^8 cfu/ml. The seedling vigour of the *Pseudomonas fluorescens* treated seeds were calculated by the standard roll towel method (ISTA, 1993). Rice seeds were gently shaken in *P. fluorescens* suspension prepared in sterile distilled water for 24 h. Seeds soaked in sterile distilled water served as control. After 24 h, seeds were blot dried, plated on wet blotters and incubated in growth chamber for 10 days. The germination percentage of seeds was calculated. The root and shoot lengths of individual seedlings were also measured to work out the vigour index using the following formula.

Vigour index = Per cent Germination x seedling length (shoot length + root length) (Abdul Baki & Anderson, 1973). The experiment was carried out with three replicates of 25 seeds each.

3.5. Greenhouse study

The bacterial strains either singly or as mixture (with and without chitin) were assessed for their efficiency in controlling sheath blight and leaffolder incidence in rice under greenhouse conditions. A pot culture study was undertaken with the following treatments by using completely randomized design (CRD) design with three replications. The different treatments are listed below

Plate 1. Oviposition cage

Plate 1



- T1: Pfl
- T2: Pfl with chitin
- T3: FP7
- T4: FP7 with chitin
- T5: Pfl+FP7
- T6: Pfl+FP7 with chitin
- T7: Carbendazim / Chlorpyrifos
- T8: Control

The talc-based bacterial formulation prepared by the following methods as described in 3.2.3.1, 3.2.3.2 and 3.2.3.3 was used in all the treatments mentioned above.

3.5.1. Seed treatment

The rice seeds were surface sterilized with 2% sodium hypochlorite and soaked in double volume of sterile distilled water containing talc-based formulation (10 g kg^{-1} of seed). After 24 h, the bacterial suspension was drained off and the seeds were dried under shade for 30 min. The seeds were allowed to sprout for another 24 h before sowing (Vidhyasekaran *et al.*, 1997b; 1999).

3.5.2. Seedling root dip

After 25 days, the seedlings were pulled out from the pots and roots of rice seedling in bundles (approximately 200 seedlings) were dipped in water containing talc formulation (20 g l^{-1}) for 2 h ensuring that the roots alone were immersed in the inoculum and then transplanted in pots at the rate of four seedlings per hill and five hill per pots (Nayar, 1996).

3.5.3. Soil application

Five g of talc-based formulation per pot was added 30 days after planting (40-45 cm height)

3.5.4. Foliar spray

The talc-based product was dissolved in water (20 g l⁻¹) and allowed to settle for 1 h, filtered through muslin cloth and the filtrate was sprayed 30 days after planting. (Vidhyasekaran *et al.*, 1999).

3.5.5. Carbendazim / Chlorpyrifos foliar spray

Plants raised from seeds treated with carbendazim (4 g kg⁻¹ of seeds) as well as 0.1% foliar spray at 30 days after planting and foliar spray with 0.04 % chlorpyrifos served as chemical check, wherever needed foliar spray with distilled water was carried out in the control treatment.

Control plants without bacterial treatment was also maintained. All the treatments were replicated three times in CRD.

3.5.6. *R. solani* challenge inoculation and disease assessment

Forty days after planting, sclerotia of *R. solani* from Petri dishes were placed (two per tiller) in the sheath of the rice plants. The inoculated portion of the plants were covered with absorbent cotton and tied with parafilm. Sheaths were regularly made damp by moistening the cotton with equal quantity of sterile water so as to maintain high humidity. The symptom development was observed after seven days and graded 0-5 scale based on the lesion size (Sriram *et al.*, 1997). Disease index was calculated using the formula,

Disease index = Total grade points × 100/ Maximum grade × No. of sheaths observed

3.5.7. Leaf folder incidence

For assessing the efficacy of *Pseudomonas* treatments against rice leaf folder, larvae of second instar were carefully collected from the cage by using the camel hair brush and released on to the leaves of treated plants and allowed to feed for three days. The per cent leaf area damaged were calculated using the formula

Per cent leaf area damage = (Leaf area consumed / Total leaf area) × 100

The total leaf area and the consumed leaf area were measured by using portable leaf area meter (Laser area meter, CI-203 model, USA).

3.5.8. Insect Bioassay

For testing the insect feeding preference under *in vitro*, detached leaves collected from the *Pseudomonas* treated plants were used. The rice leaves (1.5 g) were cut and kept in Petri plates (9-cm-diameter) lined with damp filter papers to maintain the humidity. The second instar larvae were collected from the mass rearing cage, weighed their initial weight, starved for 5 h and then allowed for feeding the leaves. The plates were incubated at $25 \pm 2^\circ\text{C}$ in the dark in 70% relative humidity for three days. After 72 h, reduction in leaf weight, larval mortality, larval and pupal weight and adult malformations were estimated for all the treatments (Maqbool *et al.*, 1998).

3.6. Assay of PGPR induced proteins

3.6.1. Sample Collection

Leaves (leaffolder) and sheath (pathogen) from uninoculated plants, maintained under the same conditions as inoculated plants were used as controls and collected at 0 h, 24 h, 48 h, 72 h, 96 h, 120 h and 7 days intervals. The leaves and sheaths of plants subjected to insect feeding or fungal infection were collected at the time intervals as mentioned above.

3.6.2. Enzyme extraction

The leaf (with and without inoculation of leaffolder) and sheath (with and without inoculation of *R. solani*) tissues were collected from bacterized and control rice plants and immediately homogenized with liquid nitrogen. One g of powdered sample was extracted with 2 ml of 0.1 M sodium citrate buffer (pH 5.0) at 4°C . The homogenate was centrifuged for 20 min at 10000 rpm. Protein extract prepared from leaves and sheath separately were used for estimation of chitinase, peroxidase (PO), polyphenol oxidase (PPO) and L-phenylalanine ammonia-lyase (PAL). The supernatant was used as crude enzyme extract for assaying chitinase activity. Sodium phosphate buffer 0.1 M (pH 7.0) was used for the extraction of PO, PPO, and PAL enzymes.

3.6.3. Determination of protein content

Protein was determined by the method of Bradford (1976). Ten mg of Coomassie brilliant blue G-250 was dissolved in 4.7 ml of absolute alcohol and 10 ml of concentrated phosphoric acid and the volume was made upto 100 ml with distilled water. A sample of 50 μ l was added to 950 μ l 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.

3.6.4. Assay of 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).

3.6.4.1. Assay procedure

The reaction mixture consisted of 10 μ l 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 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 μ l of 1M potassium phosphate buffer (pH 7.0) and incubated with 20 μ l 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 μ l 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. 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/g fresh weight.

3.6.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

One g of powdered rice leaf and sheath sample was extracted with one ml of 0.1 M sodium phosphate buffer (pH 7.0) under 4°C. The homogenate was centrifuged for 20 min

at 10000 rpm. The supernatant was used for the SDS-PAGE (Laemmli, 1970). The protein content of the sample was determined by Bradford method (Bradford, 1976).

Hundred μ g of protein from different treatments was taken and mixed with 10 μ l of sample buffer in 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) (Sigma-Aldrich Techware system, Sigma, USA). The medium range molecular weight markers (Bangalore Genei, India) were used. Electrophoresis was carried out at constant voltage of 75 volts. The gels were stained with 0.2% Coomassie brilliant blue (R250) solution. Based on the R_f value of each protein band stained, the molecular weight was calculated.

3.6.6. Western blotting

After SDS-PAGE, the proteins were electroblotted onto 0.45 μ m 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 (140 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 ($28 \pm 2^\circ\text{C}$) in TBST containing 2.5% (w/v) gelatin. The membrane was then soaked in the diluted primary antibody (barley chitinase, β -1,3-glucanase and bean TLP antisera were the gifts received from Dr. S. Muthukrishnan, Kansas State University, Manhattan, Kansas 66506 USA) (1:3000 dilution) for 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 at a dilution of 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).

3.6.7. Purification of chitinases

The sheath and leaf tissues (IR 50) from the PGPR treated rice plant challenged with *R. solani* and *C. medinalis* was ground in phosphate buffer (0.1 M; pH 7.0), and centrifuged at 6000 g for 10 min (Sorvall SUPER T 21; at 4 °C; 10,000g for 20 min.).

3.6.7.1. Ammonium sulphate precipitation

The supernatant was then precipitated with 60% $(\text{NH}_4)_2\text{SO}_4$, and the pellets were obtained after centrifugation at 12000 g for 20 min at 4°C. The pellets obtained were dissolved and resuspended in minimal amount of distilled water and dialysed overnight at 4°C against 50 mM sodium acetate buffer pH 5.5.

3.6.7.2. Gel filtration

The dialysed enzyme solution was applied onto a Sephadex G-100 column previously equilibrated with 50 mM sodium acetate buffer (buffer A) at pH 5.5. The column was eluted with the same buffer at a flow rate of 35 ml.h⁻¹. The peak fractions were pooled and concentrated by lyophilization. The lyophilized peak fraction (2ml) from the proceeding steps was applied to a Sephacryl S-300 column (1 cm x 30 cm) previously equilibrated with 50 mM phosphate buffer pH 6.0 (buffer B) containing 0.15 M sodium chloride. The column was eluted with buffer B at a flow rate of 35 ml per h. Series of five ml fractions were collected. The peak fractions were pooled and subjected to chitin affinity chromatography.

3.6.7.3. Chitin affinity chromatography

The lyophilized peak fraction 2 ml from the proceeding step was mixed in a beaker with sodium phosphate buffer (SPB)-equilibrated colloidal chitin. After 4 h of mixing, the chitin was washed three times with 30 ml of SPB, followed by three more washes with 30 ml of 25 mM sodium citrate, pH 4.0 in a beaker. The chitin was then placed in a column and eluted with 20 mM acetic acid, pH 3.2 (Swegle *et al.*, 1992).

Fractions of acetic acid elute (10ml) were collected. Protein content of all fractions was measured by the method of Bradford, (1976).

The pooled peak fractions were concentrated by lyophilisation. The lyophilized chitinase samples were separated in 12% SDS-PAGE and presence of chitinase was detected by Western blotting (3.7.7.1) with barley chitinase antiserum.

3.6.7.4 Antifungal activity of purified chitinase against phytopathogenic fungi

The assay was carried out in 9cm dia. Petri plates containing 15 ml of potato dextrose agar medium. For assays, three sterile filter paper discs of 0.6 mm diameter were placed radially in the Petri dish (2 cm away from the centre). A mycelial disc of *R. solani* was placed at the centre. The plates were incubated at room temperature until the mycelial growth from the central disc had reached nearer to the filter discs. The purified chitinase was tested for its antifungal activity against pathogenic fungi *R. solani* in agar plate at 20 µg concentration, the crude enzyme extract at 40 µg concentration and buffer served as control.

3.6.8. Peroxidase (PO)

Peroxidase activity was assayed spectrophotometrically (Hartee, 1955). The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1% H₂O₂. The reaction mixture was incubated at room temperature ($28 \pm 2^\circ \text{C}$). The change in absorbance at 420 nm was recorded at 30 sec intervals for 3 min. The boiled enzyme preparation served as blank. The enzyme activity was expressed as change in the absorbance of the reaction mixture/min/g on fresh weight basis (Hammerschmidt *et al.*, 1982).

3.6.9. Polyphenoloxidase (PPO)

Polyphenoloxidase activity was determined as per the procedure given by Mayer *et al.* (1965). One g of rice leaves or sheath were used for PPO estimation. The reaction mixture consisted of 1.5 ml of 0.1M sodium phosphate buffer (pH 6.5) and 200 µl of the enzyme extract. To start the reaction, 200 µl of 0.01 M catechol was added and the activity was expressed as changes in absorbance (in units) $\text{min}^{-1} \text{g}^{-1}$ fresh weight of tissue.

3.6.10. Activity gel electrophoresis

3.6.10.1 Peroxidase

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. After electrophoresis, the gels were incubated in the solution containing 0.15% benzidine in 6% NH_4Cl for 30 min in dark. Then 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).

3.6.10.2. Polyphenol oxidase (PPO)

The PPO is extracted by homogenizing one g of tissue in 0.01 M potassium phosphate buffer (pH 7.0). The homogenate is centrifuged at 20,000 g for 15 min. at 4°C in a centrifuge and the supernatant is used as the enzyme source. After native electrophoresis the gel was equilibrated for 30 min in 0.1% *p*-phenylene diamine in 0.1M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer. The addition of catechol was followed by a gentle shaking which resulted in appearance of dark brown discrete protein bands

3.6.11. Assay of phenylalanine ammonia-lyase (PAL)

The PAL assay was conducted 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 1mM L-phenylalanine was incubated for 60 min. 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/g fresh tissue.

3.6.12. Lignin estimation

The lignin content of leaf and sheath tissues was gravimetrically estimated following the method of Chesson, (1978). One g of the tissue (15 days after pathogen inoculation) was shaken in a mixture of 5 ml of conc. H_2SO_4 (96-98%) and 50 ml of HCl (37%) for 16 h at 25°C in a shaker (Zadrazil and Brunnet, 1986). The mixture was then transferred into one litre flask with 450 ml of distilled water. After boiling for 10 min, the content of the flask was filtered through a Geena G3 glass filter. The acidic residues were washed to neutrality with distilled water, dried at 105°C and weighed. The results were expressed in terms of per cent lignin content on dry weight basis of the tissues.

3.6.13. Phenol content

Phenol content of rice leaves was estimated as per the procedure given by Zieslin and Ben-Zaken (1993). One g of tissue was homogenized in 10 ml of 80% methanol and agitated for 15 minutes at 70°C. One ml of the methanolic 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 ml 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 GS5703 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 ($\text{C}_6\text{H}_6\text{O}$) and expressed as catechol equivalents g^{-1} tissue weight.

3.7. Field evaluation of talc formulation of individual and strain mixture on sheath blight and leaf folder incidence and on grain yield.

The talc-based formulation of individual and mixture of *Pseudomonas* strains were further tested under field conditions to study their efficacy for large-scale adoption. Two field trials were conducted with three replications in randomized complete block design during 1999. In both the trials the same treatments mentioned below were followed.

1. PF1
2. PF1 with chitin
3. FP7
4. FP7 with chitin
5. PF1+FP7
6. PF1+FP7 with chitin
7. Carbendazim
8. Chlorpyrifos
9. Carbendazim+Chlorpyrifos
10. Control

One experiment was conducted at Pondicherry and second at Paddy Breeding Station, TNAU, Coimbatore which are endemic locations for sheath blight and leaf folder incidence. Standard plot size of $5 \times 4 \text{ m}^2$ was maintained for all the treatments. Seed treatment, seedling root dipping, soil application and foliar spray of PGPR strains were done as described by Vidhyasekaran *et al.* (1999); Nandakumar *et al.* (2000). Rice seeds were soaked for 24 h in water containing talc-based formulation (10 g kg^{-1} of seeds) of individual or mixture of PGPR strains. The seeds soaked in distilled water alone served as control. Treated seeds were allowed to sprout and sown in nursery bed. The seedlings were pulled out 25 days after sowing and planted in the main field. For seedling root dipping, rice seedlings in bundles were dipped in water containing talc powder formulation (20 g l^{-1}) for 2 h and then transplanted. The seedlings were transplanted (four-seedling hill $^{-1}$) to give 66 hills m^{-2} . After draining the water from the field, the talc-based powder product (2.5 kg ha^{-1}) was mixed with 50 kg of sand and broadcasted 30 days after planting as soil application. For foliar spray, the talc-based product was dissolved in water (20 g l^{-1}) and allowed to settle for 1 h, filtered through muslin cloth and the filtrate was sprayed 30 days after planting. The efficacy of PGPR strains was assessed by comparing with the plants raised from the seeds treated with carbendazim (4 g kg^{-1} seeds), foliar spray of carbendazim (0.1%) and 0.02 % chlorpyrifos at 30 days after planting. Untreated control was also maintained. Recommended dose of fertilizer (120:38:38 kg of NPK ha^{-1}) was applied to the field.

Natural incidence of sheath blight and leaffolder damage was recorded in each plot from 40 days after planting (Vidhyasekaran *et al.*, 1999). Further, the numbers of leaffolder adults were recorded per m² area in rice field at 60 days after planting. The grain yield was recorded at the time of harvest for all treatments.

3.7.1. Occurrence of natural enemies

The occurrence of two natural enemies of leaffolder (hymenopterous parasitoid and spider) was observed in Trial II. The hymenopterous parasitoids were sampled using sweep net method at 45 days after planting while the spider numbers were counted in ten hills at two stages (45 and 55 days after transplanting).

3.8. Bacterial growth in modified medium

Selected strains of PGPR were grown on medium containing combinations of with or without chitin (1%), peptone (2%) and glycerol (1%) with continuous shaking for 48 h at room temperature ($28 \pm 2^\circ\text{C}$). Later, one ml of medium was taken and the cells were collected by centrifugation at 6000 rpm for 5 min and were resuspended in one ml of 0.1 M phosphate buffer. The population of the bacteria was calculated spectrophotometrically at 595 nm (Thompson, 1996).

3.9. Chitinase production by different bacterial strains on different media

The colorimetric assay of chitinase was carried out according to the procedure developed by Boller and Mauch (1988) as described in 3.6.4.

Statistical analyses

The data on disease severity, leaffolder incidence, natural enemies and yield were analyzed independently. The disease and pest incidence data were arcsine transformed before analysis. The treatment means were compared by Duncan's Multiple Range-Test (DMRT) (Gomez and Gomez, 1984). The package used for analysis was IRRI STAT version 92-1 developed by the International Rice Research Institute Biometrics unit, the Philippines.

Experimental Results

CHAPTER IV

EXPERIMENTAL RESULTS

4.1. Effect of *Pseudomonas fluorescens* formulation on seed germination and seedling vigour

Rice seeds when treated with the different bacterial formulations, improvement in plant growth parameters over untreated seeds was observed (Table.1). Both the strains (PF1+FP7) either with or without chitin amendment increased root and shoot length significantly. However, the increase in mean root length 20.6 cm and the mean shoot length 11.7cm due to the strain PF1 with chitin was significantly higher compared to the seedlings from untreated seeds. Similar effect was observed in all the *Pseudomonas* - treated plants also. Among the treatments, PF1 with chitin recorded higher seedling growth in all the varieties. The variety IR 50 showed the increased root and shoot length when compared to other variety (Plate 2). Bacterial formulations from the chitin amended medium comparatively improved growth of the seedlings. Such treatment recorded higher shoot and root length, although they were not significantly different from the bacterial strains without chitin. Both the strains with or without chitin amendment increased vigour index significantly. The maximum of 3125 vigour index was observed in IR 50 seedlings raised from PF1+chitin treated seeds. (Plate 3).

4.2. Effect of *P. fluorescens* formulation on sheath blight and leaffolder incidence under glasshouse conditions

4.2.1. Sheath blight incidence under glasshouse conditions

The PGPR strains and their mixture (with and without chitin amendment) were evaluated against pathogen, *R. solani* and insect, *C. medinalis*. Sheath blight development was decreased in the plants treated with PGPR strains individually and in mixture in all the three replications. Mean disease severities from the experiment were 75.22% for the non-bacterized disease control and 37.83 and 40.27% for strains PF1 and FP7 treatments respectively. The application of talc-based formulation of *Pseudomonas* strains either individually or as mixture significantly reduced the sheath blight incidence under

Table: 1. Effect of *P. fluorescens* formulation on seed germination and seedling growth

Treatments	Shoot length	Root length	Germination%	Vigour Index
CR 1009				
PF1	7.75 ^b	13.70 ^c	96.66(80.48)	2073.36 ^c
PF1+chitin	8.45 ^b	15.08 ^c	96.66(80.48)	2274.41 ^a
FP7	6.50 ^c	13.315 ^d	93.00(76.98)	1842.80 ^d
FP7+chitin	7.76 ^b	13.77 ^d	96.50(80.30)	2077.65 ^b
Control	6.21 ^b	12.30 ^d	93.00(76.98)	1721.43 ^e
TN1				
PF1	7.65 ^{bc}	19.56 ^a	96.66(80.49)	2630.12 ^b
PF1+chitin	8.04 ^b	20.35 ^a	96.66(80.49)	2744.18 ^a
FP7	7.57 ^b	17.34 ^b	100.00(85.95)	2491.00 ^c
FP7+chitin	7.75 ^b	18.55 ^b	90.11(71.91)	2369.90 ^d
Control	5.84 ^b	13.41 ^c	93.33(75.03)	1796.60 ^e
White Ponni				
PF1	6.61 ^c	17.92 ^b	100.00(85.94)	2453.00 ^b
PF1+chitin	5.54 ^c	19.25 ^b	100.00(85.94)	2479.00 ^a
FP7	5.66 ^c	15.71 ^c	96.66(80.48)	2065.62 ^d
FP7+chitin	5.92 ^c	16.44 ^c	96.66(80.48)	2161.32 ^c
Control	5.16 ^b	14.49 ^b	96.66(80.48)	1899.37 ^e
IR 50				
PF1	10.77 ^a	19.14 ^a	96.66(80.48)	2891.10 ^c
PF1+chitin	11.77 ^a	20.56 ^a	96.66(80.48)	3125.02 ^a
FP7	10.69 ^a	18.74 ^a	89.83(71.67)	2643.70 ^d
FP7+chitin	11.67 ^a	20.60 ^a	93.33(75.03)	3011.76 ^b
Control	9.71 ^a	16.37 ^a	89.66(71.53)	2338.33 ^e

The data in the parentheses are arcsine transformed values.

Values are mean of three replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

CD for vigour index x variety = 1.650

CD for shoot length x root length x variety = 1.046

CD for germination x variety = Non significant

Plate 2. Effect of *P. fluorescens* (with or without chitin) on seed germination and seedling vigour in different rice varieties

Plate 2

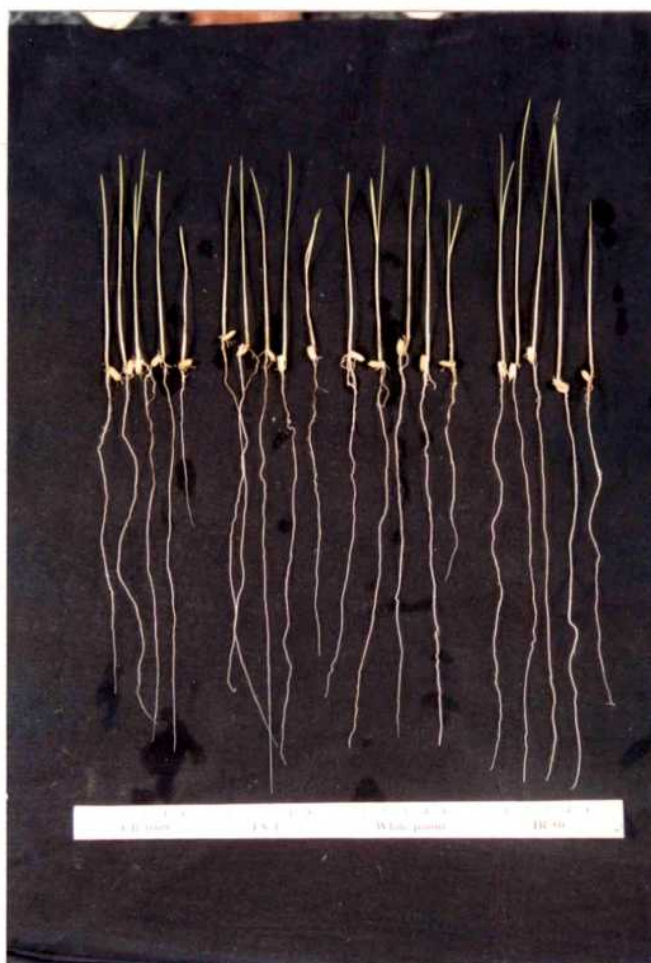


Plate 3. Effect of individual strains and strain mixtures of *P. fluorescens* on plant growth under glasshouse conditions

- 1. Control**
- 2. PF1**
- 3. PF1+chitin**
- 4. PF1+FP7**
- 5. PF1+FP7+chitin**
- 6. FP7**
- 7. FP7+chitin**

Plate 3



glasshouse conditions. Maximum of 63 per cent disease reduction was observed in plants treated with mixture of *Pseudomonas* formulations containing chitin which was statistically equal as that of the fungicide check viz., carbendazim (Table 2). However, the application of *Pseudomonas* formulation containing chitin slightly increased the level of protection offered by the bacterial strains (Plate 4).

4.2.2. Leaffolder infestation under glasshouse conditions

The leaf area damage was considered for testing the effect of the *Pseudomonas fluorescens* on rice plant. The same trend was observed for the leaffolder incidence unlike as in case of sheath blight, the maximum reduction of damage (61.6%) was observed in chemical (chlorpyrifos) treatment followed by *Pseudomonas* strain mixture 57.01% (Table 2). The mixture of the two strains PF1+FP7 with and without chitin recorded significant reduction in the leaf damage of 57 and 56 per cent respectively. Although the application of chitin amended formulation of fluorescent pseudomonads did not reduce the per cent leaf area damage by leaffolder significantly than the bacterial formulation without chitin it decreased the percent leaf area damage to some extend.

4.3. Leaffolder bio assay

4.3.1. Larval feeding preference and reduction in leaf weight

Feeding rate of leaffolder was greatly affected by both *Pseudomonas* and insecticide treatment. Generally the leaffolder larvae did not prefer the treated leaves. It was visualized by observing the feeding behaviour of larvae both in treated and control leaves. As a result, only 98 mg and 165 mg of leaves were consumed in chemical and *Pseudomonas* treated leaves respectively as against 410 mg in control treatment (Table. 3).

4.3.2. Larval mortality, reduction in larval and pupal weight

The feeding of rice leaves treated with chlorpyrifos and *Pseudomonas* caused larval mortality. An average of 86.6 % and 26.6% larval mortality was observed in chemical and strain mixture with chitin treatments respectively (Table 3). However, no

Table: 2. Effect of single and mixture of *Pseudomonas fluorescens* strains against sheath blight in rice under glasshouse conditions.

Treatments	Per cent Disease Index (PDI)	Per cent reduction of disease over control	Per cent leaf area affected	Per cent reduction of leaf area damage over control
Pfl	37.83 ^c (37.96)	49.71	19.88 ^{bcd} (26.34)	28.72
Pfl+Chitin	38.88 ^c (38.57)	48.31	18.48 ^{bc} (25.33)	33.74
FP7	40.27 ^c (39.39)	46.46	20.19 ^{cd} (26.59)	27.61
FP7+Chitin	38.61 ^c (38.41)	48.67	18.12 ^{bc} (25.16)	35.03
Pfl+FP7	34.72 ^{bc} (36.10)	53.84	12.16 ^{abc} (19.95)	56.40
Pfl+FP7 + Chitin	27.33 ^a (31.45)	63.66	11.99 ^{ab} (20.24)	57.01
Carbendazim / chllopyrphos	29.22 ^{ab} (32.71)	65.14	10.65 ^a (18.14)	61.81
Control	75.22 ^d (60.21)	-	27.89 ^d (31.85)	-

The data in the parentheses are arcsine transformed values.

Values are mean of three replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

Table: 3. Effect of *Pseudomonas* treatment on feeding behavior and development of leaffolder larvae, pupae and adults *in vitro*

Treatments	Leaf weight Consumed (mg)	Larval mortality (%)	Malformed adults emerged (%)
Pfl	317 ^c	6.7 ^a (2.37)	13.33 ^a (17.88)
Pfl+Chitin	289 ^c	6.7 ^a (2.37)	13.33 ^a (17.88)
FP7	316 ^c	6.7 ^a (2.37)	6.67 ^a (9.19)
FP7+Chitin	283 ^c	0.0 ^a (0)	6.67 ^a (9.19)
Pfl+FP7	189 ^b	20.0 ^b (17.64)	26.67 ^{ab} (30.79)
Pfl+FP7 + Chitin	165 ^{ab}	26.7 ^b (26.10)	46.67 ^b (42.70)
Chlorpyriphos	98 ^a	86.7 ^c (73.81)	96.67 ^c (83.86)
Control	410 ^d	0.0 ^a (0)	6.67 ^a (9.19)

The data in the parentheses are arcsine transformed values.

Values are mean of three replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

Plate 4. Effect of individual strains of *P. fluorescens* and their mixture on rice sheath blight incidence under glasshouse conditions

H. Healthy

C. Control

- 1. PF1**
- 2. PF1+chitin**
- 3. FP7**
- 4. FP7+chitin**
- 5. PF1+FP7**
- 6. PF1+FP7+chitin**
- 7. Carbendazim**

Plate 4



significant difference in mortality was observed in larvae fed with the leaves from PF1 and FP7 treated plants (with and without chitin) with respect to control. The same trend of reduction in larval mortality was also reflected in the total larval weight. Highest reduction in larval weight was observed in both chemical and mixture treatment but not in single strain treated leaves (Fig. 1). However the maximum mortality was brought about by chlorpyrifos treatment followed by application of *Pseudomonas* mixture. Though the difference in pupal weight was observed numerically, it was not statistically significant. In general, chemical and *Pseudomonas* treatments produced smaller pupae than control (Plate 5).

4.3.3. Adult emergence and malformed adults

Feeding on bacteria and chemical treated leaves caused delay in adult emergence. Maximum of 96.6% and 46% adult malformation were noticed in chemical and bacterial isolate mixture treatments respectively. However 6.7% mortality was also observed even in control (Table 3).

4.4. Assay of *Pseudomonas* induced proteins

4.4.1. Induction of chitinase activity

Chitinase activity was measured in the sheath and leaf extracts from the *R. solani* and *C. medinalis* treated plants respectively. Chitinase activity remained at a low level in the healthy control of both sheath and leaf samples. In contrast, the activity was stimulated strongly by both sheath blight pathogen and leaf folder insect. Accumulation of this hydrolytic enzyme was more pronounced in the *P. fluorescens* treated rice plants. During the development of symptoms, chitinase in the infected sheath markedly accumulated with a maximum of 2.5 fold at 3 days after pathogen inoculation in the mixture treated plants than the control plants (Table 4). In particular, the increase in chitinase activity occurred to a higher amount that persisted for longer time until 124 h after inoculation. The data presented in table 4 and table 5 revealed that without inoculation of *R. solani* or *C. medinalis* there were no changes in chitinase activity in the sheath or leaves of *Pseudomonas* treated plants. The bacterial strains viz. PF1 and FP7

Fig.1. Effect of *P. fluorescens* on larval and pupal weight of *C. medianalis*

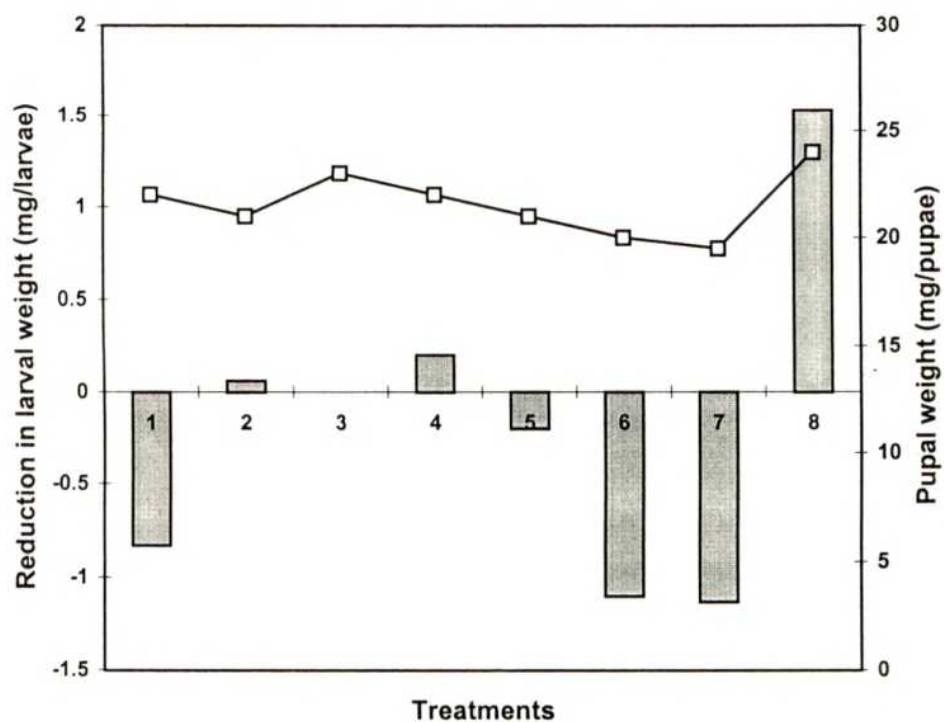


Table: 4. Effect of *Pseudomonas* treatment on chitinase activity in *R. solani* challenged plant at different intervals after infection

Treatments	Chitinase activity* in uninoculated plants (h)						Chitinase activity* in inoculated plants (h)					
	0	24	48	72	96	168	0	24	48	72	96	168
PF1	52.00 ^c	52.55 ^d	57.25 ^d	54.20 ^d	55.80 ^c	56.45 ^c	52.00 ^c	79.50 ^b	103.50 ^c	91.00 ^b	91.95 ^b	95.00 ^b
PF1+chitin	58.00 ^b	56.60 ^c	59.60 ^{cd}	58.10 ^c	59.30 ^b	57.85 ^{bc}	58.45 ^c	87.50 ^b	110.50 ^{bc}	96.65 ^{bc}	93.70 ^b	92.55 ^b
FP7	55.70 ^b	54.20 ^{cd}	57.55 ^d	57.90 ^c	56.65 ^{bc}	59.40 ^b	58.20 ^c	85.50 ^b	112.50 ^{bc}	94.66 ^{bc}	92.45 ^b	92.50 ^b
FP7+chitin	57.55 ^b	61.05 ^b	62.00 ^{bc}	61.75 ^b	59.25 ^b	58.45 ^{bc}	56.90 ^c	79.50 ^b	115.50 ^b	92.70 ^{bc}	95.80 ^b	96.35 ^{ab}
PF1+FP7	62.15 ^a	64.45 ^a	64.65 ^{ab}	66.80 ^a	62.75 ^a	65.20 ^a	68.75 ^b	119.50 ^a	134.00 ^a	134.60 ^a	107.15 ^a	105.85 ^a
PF1+FP7+chitin	62.50 ^a	64.80 ^a	64.95 ^a	66.25 ^a	62.20 ^a	62.60 ^a	85.87 ^a	127.05 ^a	140.00 ^a	102.35 ^b	108.00 ^a	105.35 ^a
Control	38.15 ^d	36.90 ^c	42.60 ^c	42.10 ^c	40.05 ^d	38.60 ^d	38.30 ^d	48.65 ^d	53.80 ^d	52.50 ^d	48.55 ^c	45.65 ^c

* μmol of GlcNAc equivalent/min/g of fresh tissue

Values are mean of two replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

CD for treatment x hour (uninoculated) : 2.686

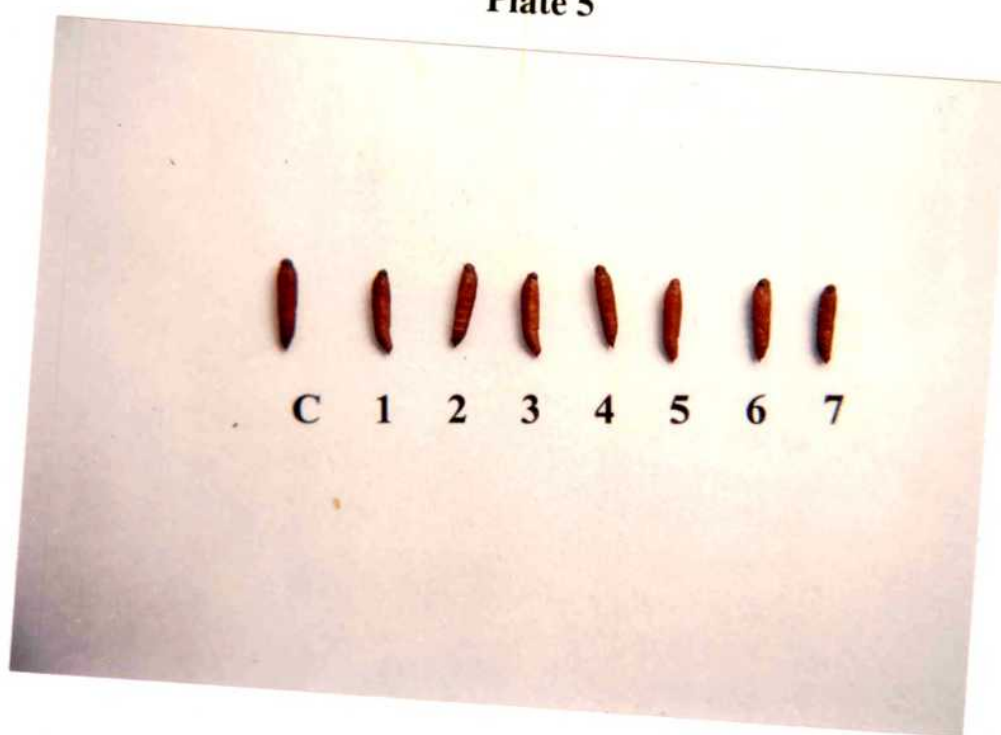
CD for treatment x hour (inoculated) : 9.468

Plate 5. Effect of individual strains and strain mixture of *P. fluorescens* treated plant on pupal size of *C. medinalis* under glasshouse conditions

C. Control

- 1. PF1**
- 2. PF1+chitin**
- 3. FP7**
- 4. FP7+chitin**
- 5. PF1+FP7**
- 6. PF1+FP7+chitin**
- 7. Chlorpyrifos**

Plate 5



induced same level of chitinase in leaves of rice plant. Maximum of 83.42% and 71.22% increased chitinase activity were noticed in the mixture treatments with and without chitin respectively (Table 5) after challenged with leaffolder as against the control plant. The results indicated that the *Psuedomonas* strain mixture stimulated chitinase activity not only locally but also systemically to the sheath and the leaves after the infection of the pathogen and insect respectively (Fig. 2, 3).

4.4.2. Induction of peroxidase activity

Peroxidase activity was significantly higher in rice sheath and leaves inoculated with *R. solani* and *C. medinalis* than the bacterized plants without challenge inoculation. In general, no significant difference of PO activity existed in the bacterized plant over time in uninoculated plants. Although there was increased PO activity in the *R. solani* and *C. medinalis* inoculated control plant, it was significantly greater when they were inoculated to the bacterized plant. The highest level 45.3% increase peroxidase activity occurred in leaffolder-challenged plants bacterized with mixture of the strains PF1 and FP7. When bacterized plants (with individual strains) were challenged with the *C. medinalis*, peroxidase activity increased rapidly within a 24 h period to levels that nearly paralleled but did not exceed those of plants bacterized with mixture. Although the timing of peroxidase expression was similar, the conditions were different in bacterized versus unbacterized control by over expression in the sheath and leaves of the bacterized plants after challenging with pathogen and insect (Table 6, 7) (Fig. 4, 5)

4.4.3. Phenylalanine ammonia-lyase (PAL) activity

Sheath and leaf samples from the *P. fluorescens* treated plants were used to test total PAL activity after challenge inoculation with *R. solani* and *C. medinalis*. Experimental results revealed that bacterial strain individually and in mixture activated PAL in rice sheath and leaf of the rice plant. Leaffolder feeding was found to induce a rapid and transient accumulation of PAL at the site of infestation than the pathogen. However, in pathogen infected portion, the PAL accumulation was less than the leaffolder-infested leaf. Moreover, PAL activity dropped to values close to those measured in untreated leaves 48 h and 72 h after leaffolder infestation and pathogen

Table: 5. Effect of *Pseudomonas* bacterization on chitinase activity in *C. medinalis* challenged plant.

Treatment	Chitinase activity* in uninoculated plants (h)						Chitinase activity* in inoculated plants (h)					
	0	24	48	72	96	168	0	24	48	72	96	168
PF1	124.35 ^c	132.30 ^c	129.05 ^b	128.40 ^c	131.75 ^{ab}	127.80 ^{bc}	122.60 ^c	170.00 ^c	181.0 ^d	157.00 ^b	131.70 ^d	135.25 ^b
PF1+chitin	124.70 ^c	122.60 ^d	106.60 ^d	114.30 ^d	126.40 ^b	126.95 ^{bc}	123.00 ^c	169.50 ^c	187.00 ^{cd}	158.00 ^b	145.45 ^c	134.80 ^b
FP7	132.20 ^c	133.80 ^{bc}	118.50 ^c	126.95 ^c	132.20 ^{ab}	121.55 ^c	119.40 ^c	177.50 ^{bc}	184.50 ^{cd}	154.00 ^b	140.05 ^{cd}	128.45 ^b
FP7+chitin	128.90 ^c	124.10 ^d	120.00 ^c	125.35 ^c	116.95 ^c	126.00 ^{bc}	129.10 ^{bc}	171.60 ^c	194.20 ^c	156.90 ^b	144.40 ^c	132.20 ^b
PF1+FP7	144.85 ^b	140.85 ^b	151.30 ^a	136.95 ^b	133.20 ^{ab}	130.15 ^b	137.80 ^{ab}	183.5 ^b	207.60 ^b	184.75 ^a	168.35 ^b	168.50 ^a
PF1+FP7+chitin	158.15 ^a	148.75 ^a	152.40 ^a	147.60 ^a	139.40 ^a	140.05 ^a	139.95 ^a	196.60 ^a	222.30 ^a	176.05 ^a	178.80 ^a	172.25 ^a
Control	72.70 ^d	77.90 ^c	79.85 ^c	74.70 ^e	74.40 ^d	71.20 ^d	72.70 ^d	121.85 ^d	120.25 ^e	128.20 ^c	121.20 ^e	107.90 ^c

* μmol of GlcNAc equivalent/min/g of fresh tissue

Values are mean of two replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple

range test at $P = 0.05$

CD for treatment x hour (uninoculated) : 7.455

CD for treatment x hour (inoculated) : 10.308

Table: 6. Changes in peroxidase activity in *R. solani* challenged bacterized plant at different intervals after infection

Treatment	PO activity* in uninoculated plants (h)					PO activity* in inoculated plants (h)						
	0	24	48	72	96	168	0	24	48	72	96	168
PF1	1.31 ^b	1.35 ^b	1.33 ^d	1.33 ^d	1.33 ^d	1.34 ^c	1.32 ^b	1.38 ^b	1.65 ^{bc}	1.82 ^b	1.65 ^{bc}	1.37 ^{bc}
PF1+chitin	1.31 ^b	1.35 ^b	1.33 ^d	1.39 ^d	1.33 ^c	1.33 ^{cd}	1.32 ^b	1.38 ^b	1.69 ^b	1.91 ^{bc}	1.70 ^{bc}	1.42 ^b
FP7	1.32 ^b	1.36 ^b	1.37 ^c	1.30 ^e	1.35 ^{de}	1.34 ^{cd}	1.31 ^b	1.40 ^b	1.59 ^c	1.81 ^b	1.71 ^b	1.32 ^{cd}
FP7+chitin	1.32 ^b	1.31 ^c	1.38 ^c	1.38 ^c	1.26 ^c	1.28 ^e	1.32 ^a	1.39 ^b	1.61 ^c	1.93 ^{bc}	1.69 ^{bc}	1.42 ^b
PF1+FP7	1.40 ^a	1.46 ^a	1.46 ^b	1.51 ^b	1.48 ^b	1.52 ^b	1.40 ^a	1.53 ^a	1.71 ^b	2.08 ^{cd}	2.06 ^a	1.58 ^a
PF1+FP7+chitin	1.43 ^a	1.45 ^a	1.50 ^a	1.52 ^a	1.51 ^a	1.51 ^a	1.41 ^c	1.51 ^a	1.80 ^a	2.19 ^b	2.06 ^a	1.60 ^a
Control	1.15 ^c	1.23 ^d	1.25 ^e	1.32 ^e	1.35 ^c	1.33 ^{cd}	1.12 ^c	1.33 ^b	1.51 ^d	1.90 ^e	1.57 ^d	1.31 ^{cd}

* Abs/min/g of fresh tissue

Values are mean of two replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

CD for treatment x hour (uninoculated) : 0.039

CD for treatment x hour (inoculated) : 0.060

Table: 7. Changes in peroxidase activity in *C. medinalis* challenged bacterized plant at different intervals after infestation

Treatment	PO activity* in uninoculated leaves (h)						PO activity* in inoculated leaves (h)					
	0	24	48	72	96	168	0	24	48	72	96	168
PF1	1.68 ^a	1.68 ^a	1.67 ^a	1.68 ^a	1.67 ^a	1.65 ^a	1.69 ^{cd}	2.55 ^d	2.14 ^{cd}	1.89 ^b	1.80 ^b	1.72 ^{cd}
PF1+chitin	1.73 ^a	1.73 ^a	1.74 ^a	1.73 ^a	1.73 ^a	1.72 ^a	1.73 ^{bc}	2.69 ^c	2.16 ^c	1.99 ^a	1.82 ^{bc}	1.77 ^{bc}
FP7	1.64 ^a	1.65 ^a	1.64 ^a	1.63 ^a	1.64 ^a	1.64 ^a	1.64 ^d	2.53 ^d	2.04 ^e	1.80 ^c	1.73 ^{bcd}	1.73 ^{bcd}
FP7+chitin	1.68 ^a	1.68 ^a	1.67 ^a	1.69 ^a	1.67 ^a	1.68 ^a	1.68 ^{cd}	2.65 ^c	2.09 ^{de}	1.83 ^{bc}	1.77 ^{cd}	1.71 ^{cd}
PF1+FP7	1.76 ^a	1.76 ^a	1.76 ^a	1.77 ^a	1.77 ^a	1.69 ^a	1.77 ^{ab}	2.95 ^b	2.35 ^b	2.03 ^a	1.94 ^a	1.86 ^a
PF1+FP7+chitin	1.82 ^a	1.85 ^a	1.89 ^a	1.87 ^a	1.85 ^a	1.86 ^a	1.83 ^a	3.25 ^a	2.60 ^a	2.03 ^a	1.94 ^a	1.78 ^b
Control	1.34 ^a	1.35 ^a	1.39 ^a	1.48 ^a	1.39 ^a	1.45 ^a	1.35 ^c	2.03 ^e	1.70 ^f	1.64 ^d	1.59 ^d	1.69 ^d

* Abs/min/g of fresh tissue

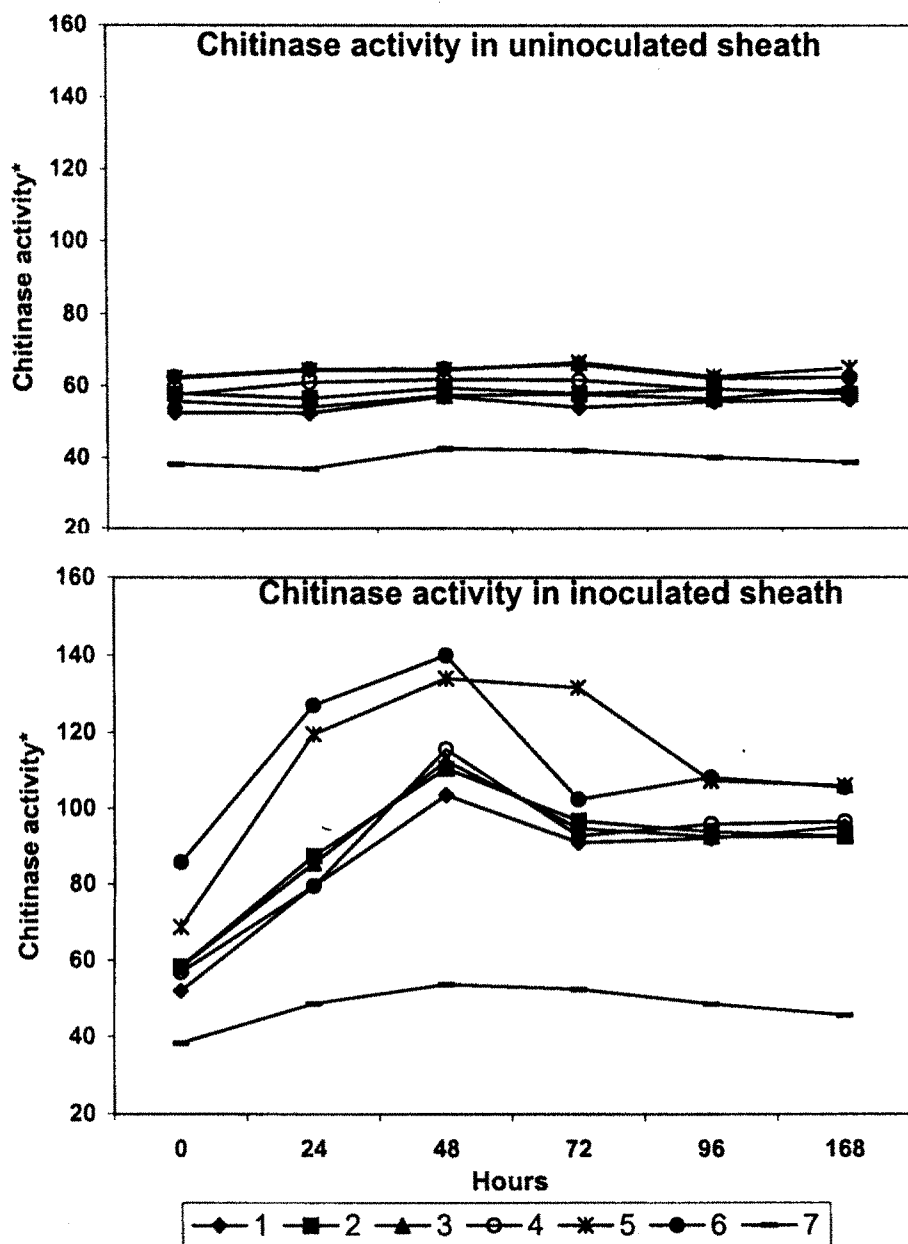
Values are mean of two replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

CD for treatment x hour (uninoculated) : Non significant

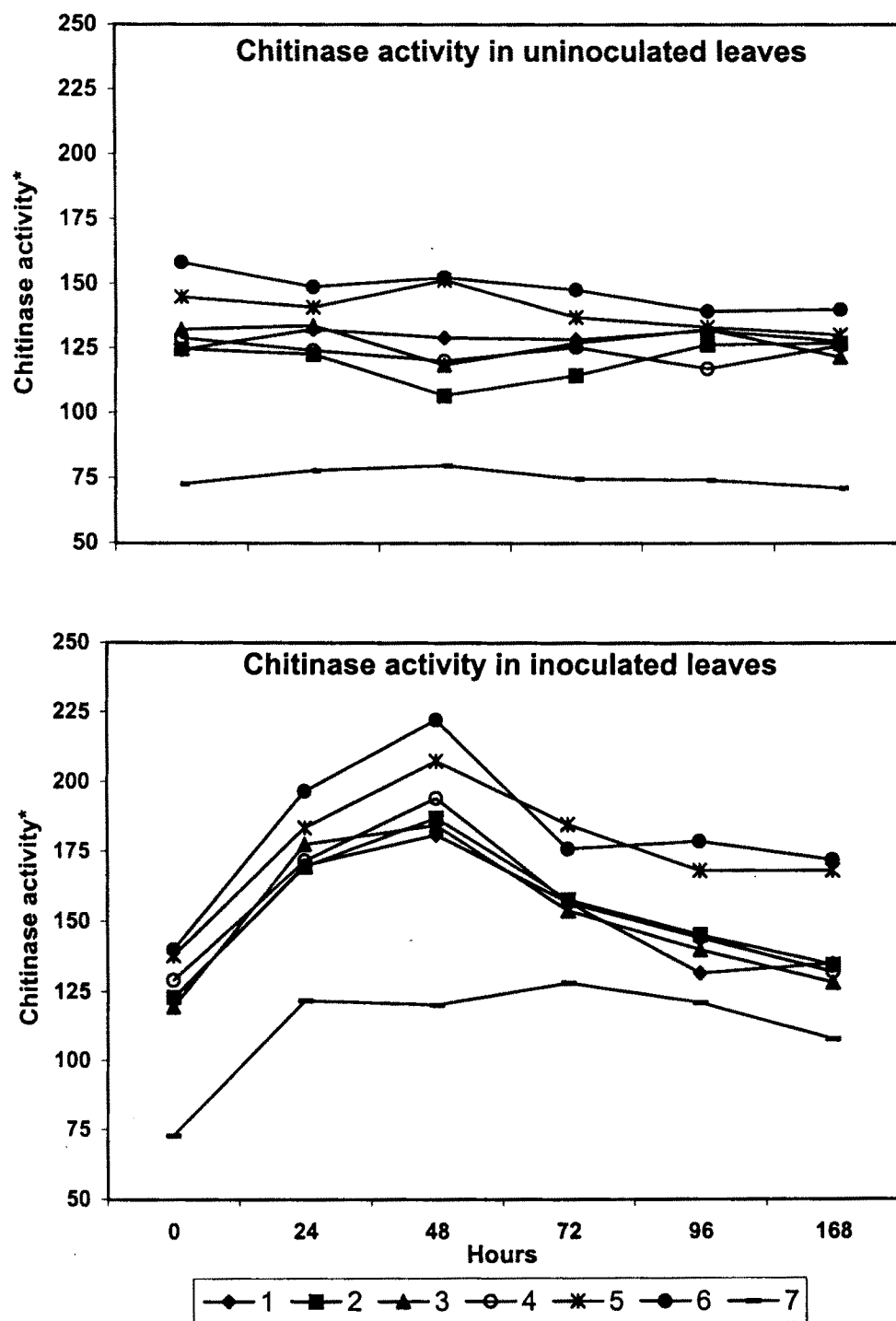
CD for treatment x hour (inoculated) : 0.061

Fig. 2. Effect of *Pseudomonas* bacterization on Chitinase activity in *R. solani* challenged plant.



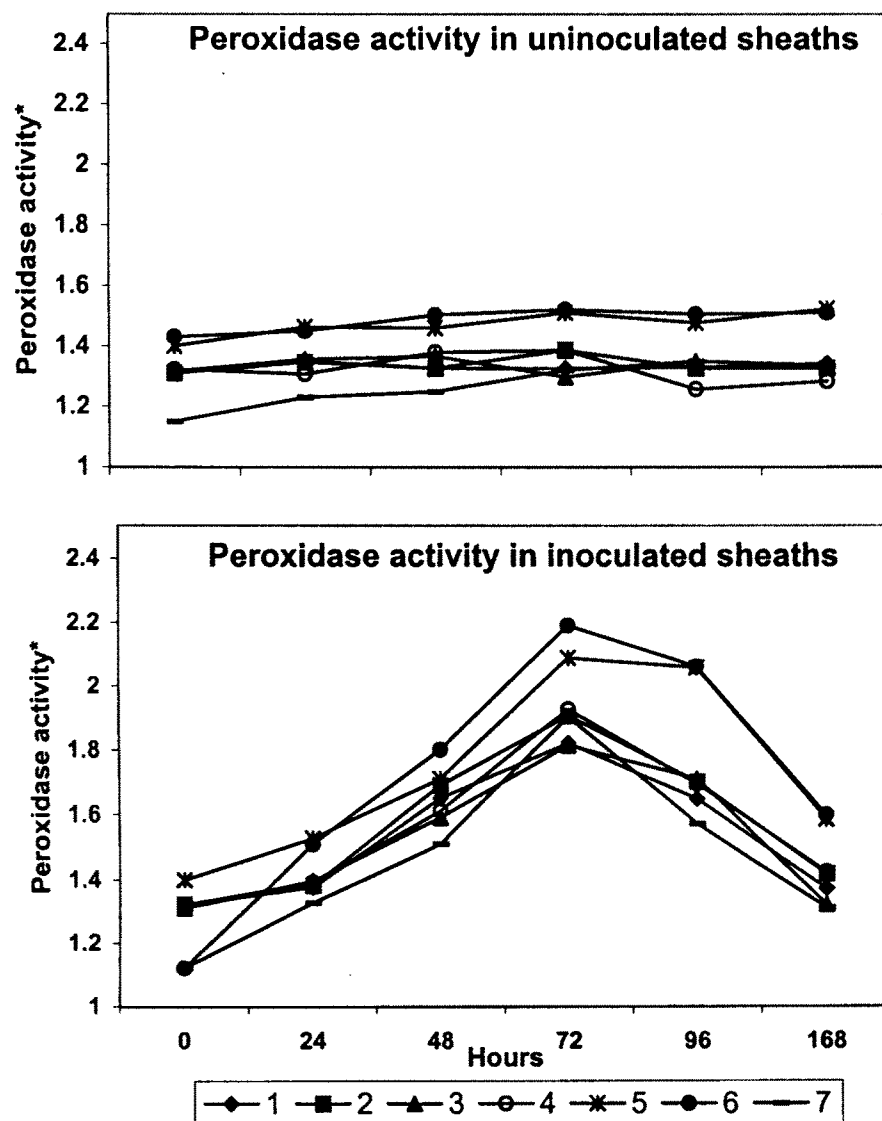
*umol of GlcNAc equivalent/min/g of fresh tissue

Fig. 3. Effect of *Pseudomonas* bacterization on Chitinase activity in *C. medinalis* challenged plant.



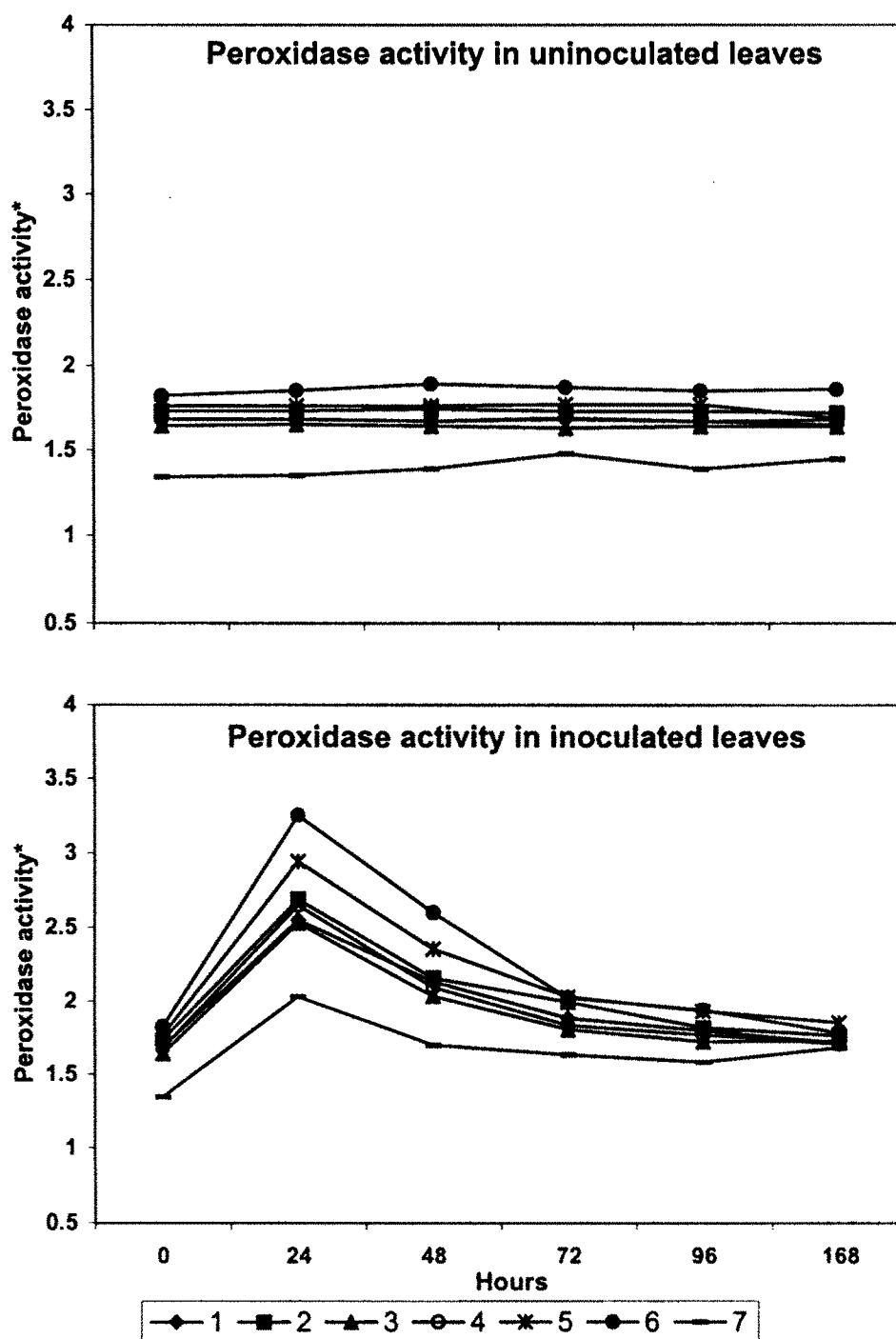
*nmol of GlcNAc equivalent/min/g of fresh tissue

Fig. 4. Effect of *Pseudomonas* bacterization on Peroxidase activity in *R. solani* challenged plant.



*Abs/min /g of fresh weight

Fig. 5. Effect of *Pseudomonas* bacterization on peroxidase in *C. medinalis* challenged plants



*Abs/min/g of fresh weight

infection respectively (Fig. 6, 7). In both set of treatments, bacterized rice plants showed significantly increased level of PAL activity than the control treatment. PAL activity began to increase by 24 h after inoculation in the pathogen and insect inoculated plant. The increase in activity was transient in the sheath and leaves and activity returned to control levels by 72 h after inoculation in the pathogen inoculated sheath. But in the case of insect, the PAL activity occurred at 24 hs after inoculation in the leaves. The increases in PAL activity in both sheath and leaves of bacterized and control rice plants were unaffected. No much difference in PAL activities were detected with and without chitin amendment. The induced activity of PAL in the sheath and leaves of bacterized rice plants inoculated with pathogen and insect was at least 2 fold higher than the control plants (Table 8, 9).

4.4.4. Induction of polyphenol oxidase (PPO) activity

Upon pathogen challenge inoculation, PPO activity was significantly increased in the bacterized rice plant than the control. PPO accumulation reached maximum at 72 h after inoculation and it was significantly higher in sheaths bacterized by *P. fluorescens*. Although PPO activity was increased significantly it was high in the mixture treatments. However, there was no difference in the chitin-amended treatment. It was inferred that application of *Pseudomonas* strains led to increase in PPO activity. Much variation among the treatments was observed. Significant induction of PPO was noticed at 24 h after pathogen inoculation and maintained the same level upto 72 h and thereafter started to decline. But in the case of control, challenge inoculation with insect significantly induced PPO activity that reached maximum at 24 h and started to decline thereafter. Maximum of 42.3 per cent increased PPO activity was detected in plants treated by PF1+FP7+chitin followed by 44.7 per cent in PF1+FP7 in both pathogen and insect challenged plants respectively. Among the two strains PF1 and FP7, the best inducer of PPO was PF1 which recorded 34.2 and 34.8 per cent increased PPO activity in pathogen and insect challenged plant respectively (Table 10, 11) (Fig. 8, 9)

Fig. 6. Effect of *Pseudomonas* bacterization on PAL activity in *R. solani* challenged plant.

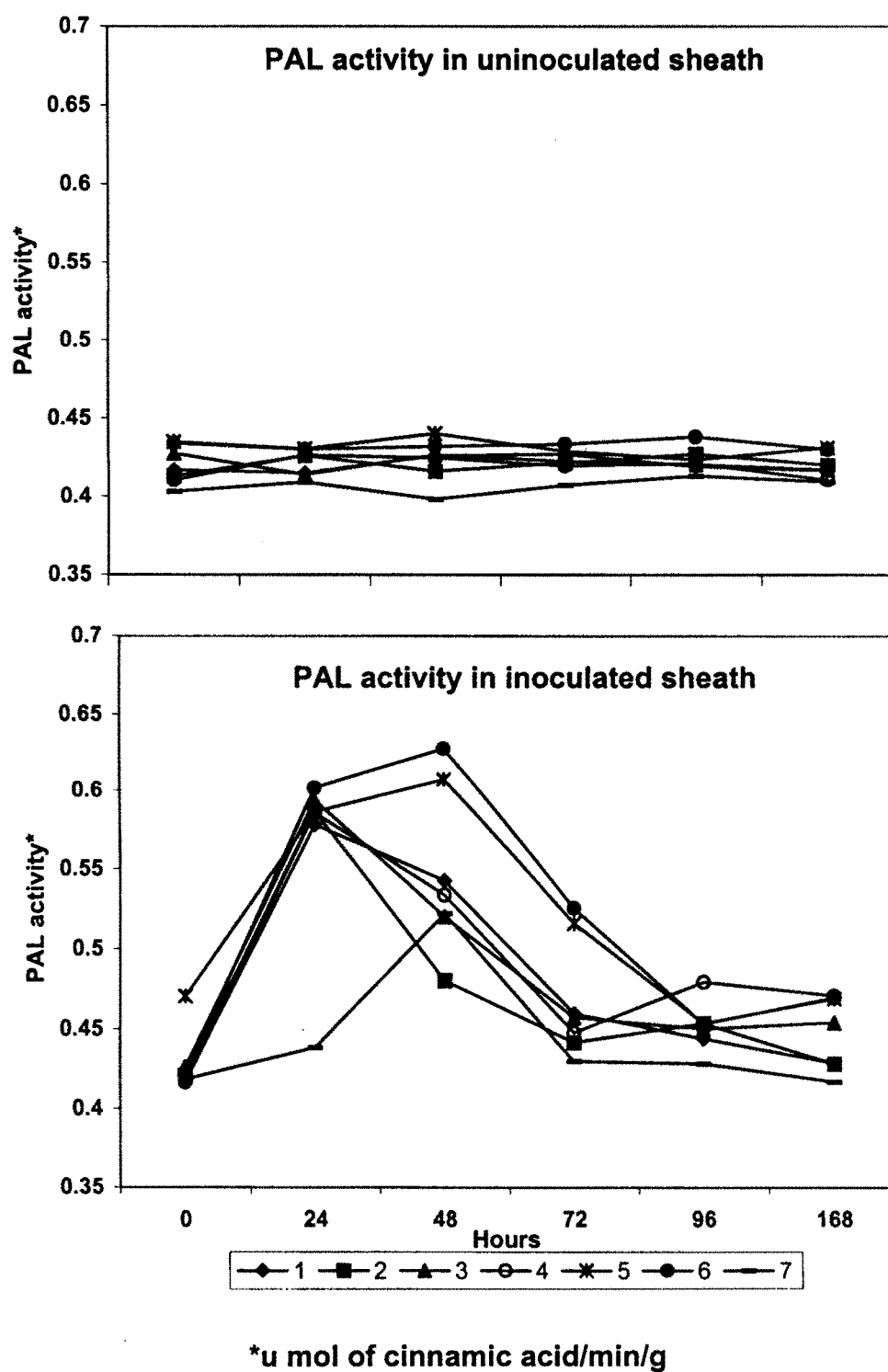


Fig. 7. Effect of *Pseudomonas* bacterization on PAL activity in *C. medinalis* challenged plant.

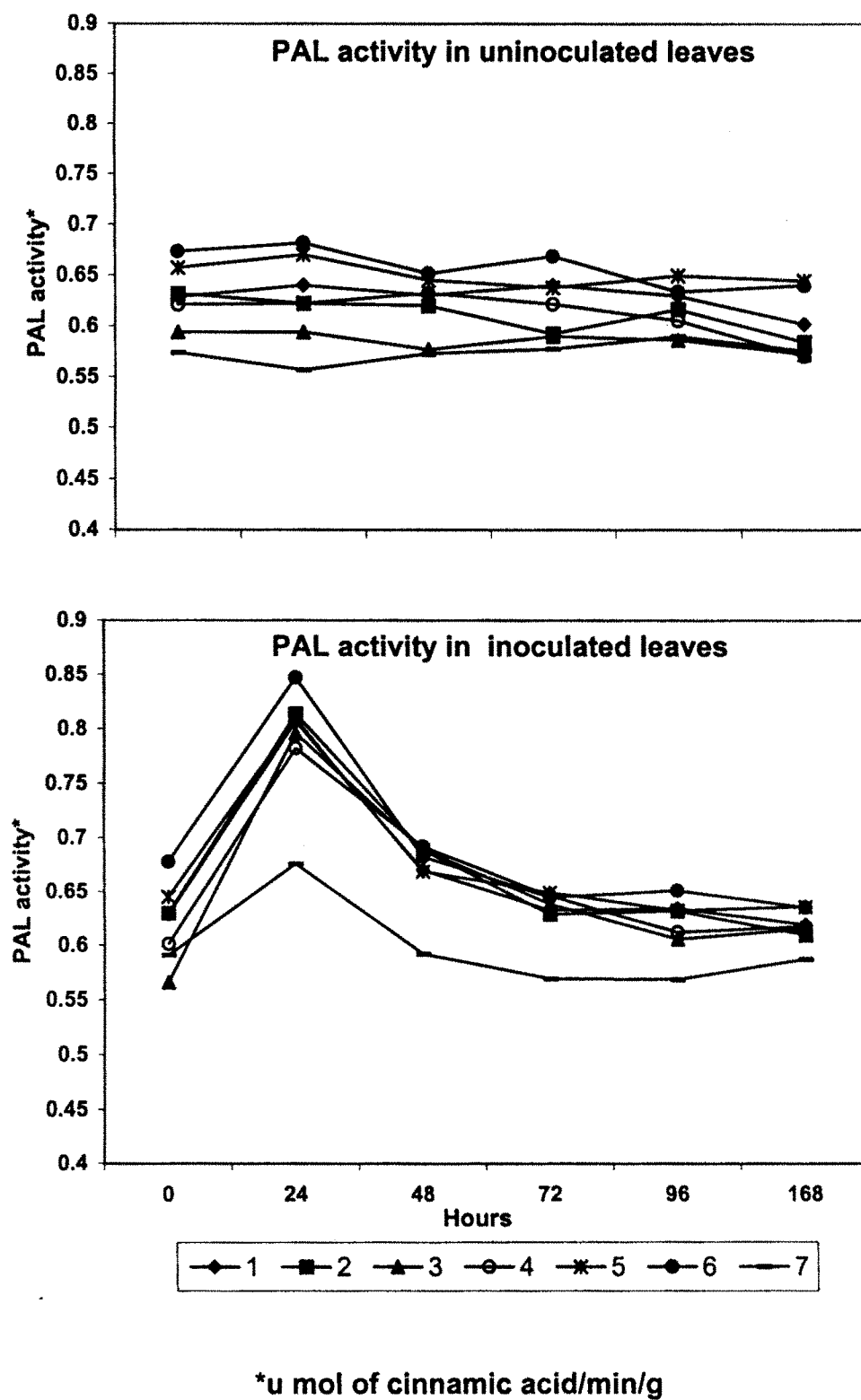


Table: 8. Effect of *Pseudomonas* bacterization on PAL activity in *R. solani* challenged plant

Treatment	PAL activity* in uninoculated plants (h)							PAL activity* in inoculated plants (h)						
	0	24	48	72	96	168		0	24	48	72	96	168	
PF1	0.42 ^{bc}	0.42 ^{ab}	0.43 ^{ab}	0.43 ^a	0.42 ^b	0.42 ^{ab}		0.42 ^b	0.58 ^a	0.54 ^b	0.46 ^b	0.44 ^{ab}	0.43 ^{bc}	
PF1+chitin	0.41 ^{bc}	0.43 ^a	0.42 ^b	0.42 ^a	0.43 ^{ab}	0.42 ^{ab}		0.42 ^b	0.59 ^a	0.48 ^c	0.44 ^b	0.45 ^{ab}	0.43 ^{bc}	
FP7	0.43 ^{ab}	0.41 ^{ab}	0.43 ^{ab}	0.42 ^a	0.42 ^b	0.42 ^{ab}		0.43 ^b	0.59 ^a	0.52 ^b	0.46 ^b	0.45 ^{ab}	0.45 ^{ab}	
FP7+chitin	0.41 ^c	0.43 ^a	0.42 ^{ab}	0.42 ^a	0.42 ^{ab}	0.41 ^b		0.42 ^b	0.59 ^a	0.53 ^b	0.45 ^b	0.48 ^a	0.47 ^a	
PF1+FP7	0.44 ^a	0.43 ^a	0.44 ^a	0.43 ^a	0.42 ^{ab}	0.43 ^a		0.47 ^a	0.59 ^a	0.61 ^a	0.52 ^a	0.45 ^{ab}	0.47 ^a	
PF1+FP7+chitin	0.43 ^a	0.43 ^a	0.43 ^{ab}	0.43 ^a	0.43 ^a	0.43 ^a		0.42 ^b	0.60 ^a	0.63 ^a	0.53 ^a	0.45 ^{ab}	0.47 ^a	
Control	0.40 ^c	0.41 ^b	0.39 ^c	0.42 ^a	0.43 ^{ab}	0.41 ^b		0.42 ^b	0.44 ^b	0.52 ^b	0.43	0.43 ^b	0.42 ^c	

*µg of cinnamic acid/min/g

Values are mean of two replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

CD for treatment x hour (uninoculated) : 0.034

CD for treatment x hour (inoculated) : 0.062

Table: 9. Effect of *Pseudomonas* bacterization on PAL activity in *C. medinalis* challenged plant.

Treatment	PAL activity* in uninoculated plants (h)						PAL activity* in inoculated plants (h)					
	0	24	48	72	96	168	0	24	48	72	96	168
PF1	0.63 ^b	0.64 ^b	0.63 ^{bc}	0.64 ^b	0.63 ^b	0.60 ^b	0.63 ^{bc}	0.81 ^{bc}	0.67 ^a	0.63 ^a	0.64 ^{ab}	0.62 ^a
PF1+chitin	0.63 ^b	0.62 ^b	0.62 ^c	0.59 ^c	0.62 ^{bc}	0.58 ^{bc}	0.63 ^{bc}	0.81 ^b	0.69 ^a	0.63 ^a	0.63 ^{ab}	0.61 ^{ab}
FP7	0.59 ^c	0.59 ^c	0.58 ^d	0.59 ^c	0.59 ^d	0.57 ^c	0.57 ^c	0.80 ^{bc}	0.69 ^a	0.64 ^a	0.61 ^b	0.62 ^{ab}
FP7+chitin	0.62 ^b	0.62 ^b	0.63 ^{bc}	0.62 ^b	0.61 ^{cd}	0.57 ^c	0.60 ^{cd}	0.78	0.69 ^a	0.65 ^a	0.61 ^b	0.62 ^{ab}
PF1+FP7	0.66 ^a	0.67 ^a	0.65 ^{ab}	0.64 ^b	0.65 ^a	0.64 ^a	0.65 ^b	0.81 ^{bc}	0.67 ^a	0.65 ^a	0.63 ^{ab}	0.64 ^a
PF1+FP7+chitin	0.67 ^a	0.68 ^a	0.65 ^a	0.67 ^a	0.63 ^{ab}	0.64 ^a	0.68 ^a	0.85 ^a	0.68 ^a	0.64 ^a	0.65 ^a	0.64 ^a
Control	0.57 ^d	0.56 ^d	0.57 ^d	0.58 ^c	0.59 ^d	0.58 ^c	0.59 ^{de}	0.68 ^d	0.59 ^b	0.57 ^b	0.57 ^c	0.59 ^b

*µg of cinnamic acid/min/g

Values are mean of two replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

CD for treatment x hour (uninoculated) : 0.018

CD for treatment x hour (inoculated) : 0.028

Table: 10. Changes in PPO activity in bacterized plant at different intervals after challenged with *R. solani*.

Treatments	Polyphenol oxidase activity* in uninoculated plants (h)							Polyphenol oxidase activity* in inoculated plants (h)						
	0	24	48	72	96	120	168	0	24	48	72	96	120	168
PF1	0.59 ^a	0.59 ^a	0.58 ^a	0.58 ^a	0.57 ^a	0.57 ^a	0.50 ^a	0.59 ^f	0.80 ^d	1.16 ^a	0.92 ^b	0.83 ^c	0.78 ^d	0.72 ^e
PF1+chitin	0.59 ^a	0.58 ^a	0.58 ^a	0.58 ^a	0.58 ^a	0.59 ^a	0.58 ^a	0.59 ^f	0.81 ^c	1.12 ^a	0.93 ^b	0.84 ^c	0.78 ^d	0.73 ^e
FP7	0.57 ^a	0.58 ^a	0.57 ^a	0.59 ^a	0.57 ^a	0.57 ^a	0.58 ^a	0.57 ^f	0.79 ^c	1.05 ^a	0.89 ^b	0.80 ^c	0.74 ^d	0.70 ^e
FP7+chitin	0.58 ^a	0.57 ^a	0.58 ^a	0.57 ^a	0.58 ^a	0.57 ^a	0.57 ^a	0.58 ^f	0.80 ^c	1.08 ^a	0.90 ^b	0.82 ^c	0.76 ^d	0.71 ^e
PF1+FP7	0.59 ^a	0.59 ^a	0.59 ^a	0.58 ^a	0.58 ^a	0.59 ^a	0.58 ^a	0.59 ^f	0.82 ^c	1.23 ^a	0.97 ^b	0.84 ^c	0.79 ^d	0.73 ^e
PF1+FP7+chitin	0.60 ^a	0.60 ^a	0.61 ^a	0.59 ^a	0.59 ^a	0.59 ^a	0.59 ^a	0.60 ^g	0.82 ^d	1.23 ^a	0.98 ^b	0.86 ^c	0.79 ^e	0.74 ^f
Control	0.49 ^a	0.48 ^a	0.48 ^a	0.48 ^a	0.47 ^a	0.48 ^a	0.47 ^a	0.49 ^e	0.65 ^c	0.86 ^a	0.68 ^b	0.65 ^c	0.61 ^d	0.59 ^d

* Abs /min/g of fresh tissue

Values are mean of two replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

CD for treatment x hour (uninoculated) : Non-significant

CD for treatment x hour (inoculated) : 0.026

Table: 11. Changes in PPO activity in *C. medinalis* challenged bacterized plant at different intervals after infestation

Treatments	Polyphenol oxidase activity* in uninoculated plants (h)						Polyphenol oxidase activity* in inoculated plants (h)							
	0	24	48	72	96	120	168	0	24	48	72	96	120	168
PF1	0.81 ^a	0.81 ^a	0.83 ^a	0.81 ^a	0.80 ^a	0.81 ^a	0.80 ^a	0.80 ^{bc}	1.44 ^b	1.26 ^b	1.07 ^b	0.93 ^b	0.88 ^a	0.82 ^{ab}
PF1+chitin	0.84 ^a	0.83 ^a	0.83 ^a	0.82 ^a	0.82 ^a	0.82 ^a	0.80 ^a	0.84 ^b	1.50 ^{ab}	1.23 ^b	1.11 ^b	0.90 ^b	0.87 ^a	0.84 ^a
FP7	0.77 ^a	0.76 ^a	0.76 ^a	0.77 ^a	0.80 ^a	0.76 ^a	0.75 ^a	0.77 ^{cd}	1.31 ^c	1.20 ^b	1.07 ^b	0.89 ^b	0.87 ^a	0.82 ^{ab}
FP7+chitin	0.79 ^a	0.79 ^a	0.80 ^a	0.74 ^a	0.80 ^a	0.79 ^a	0.77 ^a	0.79 ^{bcd}	1.32 ^c	1.25 ^b	1.10 ^b	0.90 ^b	0.89 ^a	0.85 ^a
PF1+FP7	0.91 ^a	0.90 ^a	0.90 ^a	0.85 ^a	0.88 ^a	0.79 ^a	0.82 ^a	0.91 ^a	1.52 ^a	1.41 ^a	1.33 ^a	1.08 ^a	0.90 ^a	0.89 ^a
PF1+FP7+chitin	0.96 ^a	0.94 ^a	0.95 ^a	0.89 ^a	0.79 ^a	0.80 ^a	0.86 ^a	0.96 ^a	1.54 ^a	1.43 ^a	0.83 ^d	1.10 ^a	1.92 ^a	0.89 ^a
Control	0.72 ^a	0.73 ^a	0.72 ^a	0.73 ^a	0.78 ^a	0.75 ^a	0.70 ^a	0.72 ^d	1.07 ^d	0.89 ^c	0.98 ^c	0.77 ^c	0.75 ^b	0.75 ^b

* Abs/min/g of fresh tissue

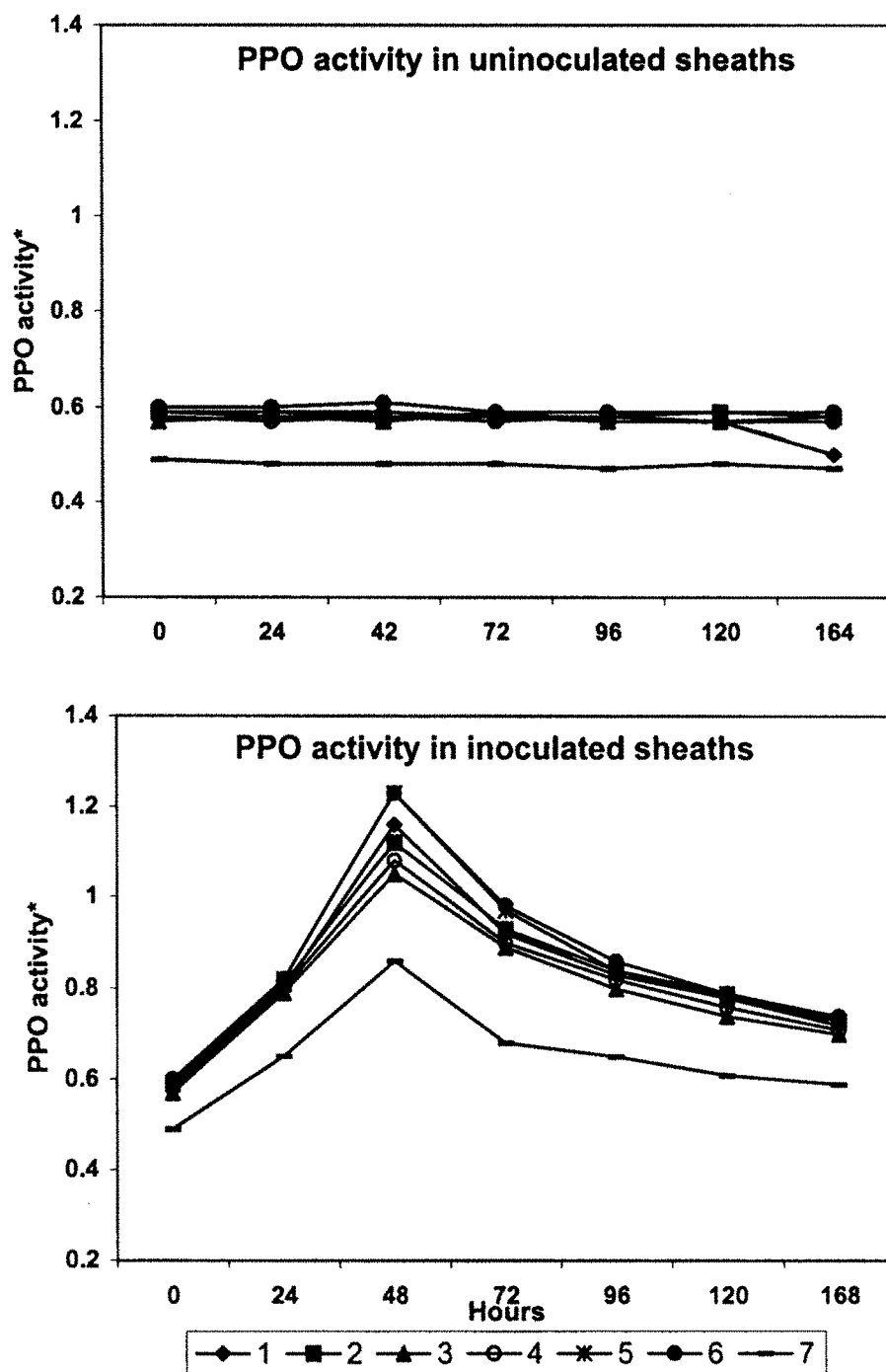
Values are mean of two replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

CD for treatment x hour (uninoculated) : Non-significant

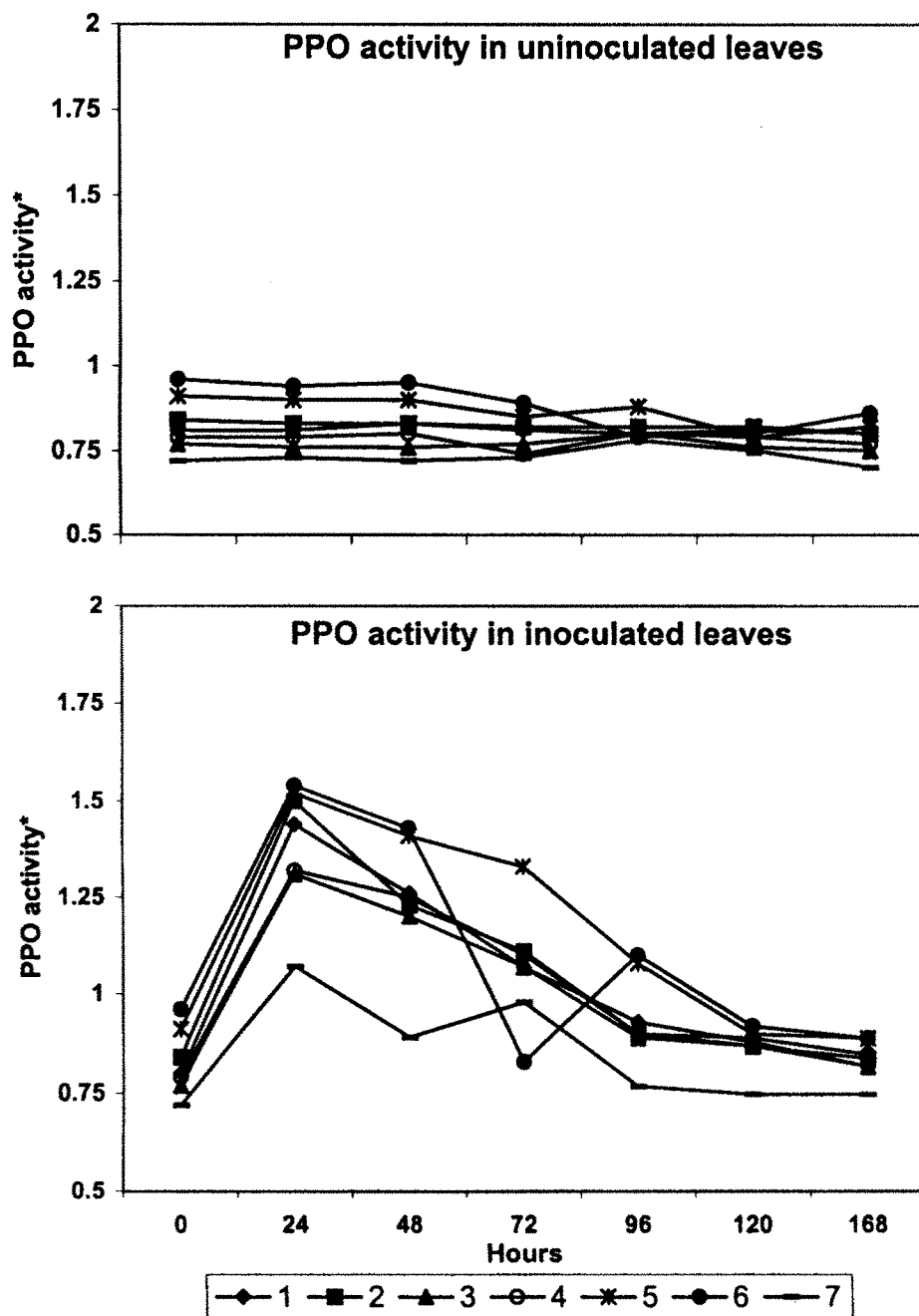
CD for treatment x hour (inoculated) : 0.186

Fig. 8. Effect of *Pseudomonas* bacterization on PPO activity in rice challenged with *R.solani*



*Abs/min/g of fresh weight

Fig. 9. Effect of *Pseudomonas* bacterization on PPO activity in rice plant challenged with *C. medinalis*



*Abs/min/g of fresh tissue

4.4.5. Phenol content

The fluorescent pseudomonads individually and in mixture differed in their abilities to stimulate defense responses in rice plant. Treatment of strain mixture to rice induced browning and the accumulation of phenol, where as the individual stain elicited a weaker response (Table 12).

4.5. Lignification

Both the leaffolder and sheath blight attacked plants showed the lignification of the leaves and sheath respectively. Increased lignin content was recorded in the *Pseudomonas* treated plants that to in the mixture treatment at seven days after inoculation on the sheath blight pathogen and leaffolder (Table 13).

4.6. SDS-PAGE analysis of crude protein of *Pseudomonas* treated plants

In the susceptible rice cultivar IR-50, *P. fluorescens* treatment induced proteins with molecular weight of 41 and 56 kDa and several new proteins appeared with high intensity. The protein with molecular weight of 56 kDa appeared to be increased with *Pseudomonas* treatment and leaffolder infestation. In *R. solani* inoculation *Pseudomonas* treated plants, proteins with molecular weight of 53 and 18 kDa had appeared to visible but less induction of this protein were noticed in control plants (Plate 6). In *C. medinalis* infested *Pseudomonas* treated plants, proteins with molecular weight of 41 kDa were detected which was not noticed with high intensity or completely absent in the challenged control plants (Plate 7, 8, 9, 10).

4.7. Isoform pattern of peroxidase

Native gel electrophoretic separation of enzyme extract from *Pseudomonas* treated plants showed different Peroxidase (PO) patterns. The bacterized plants after challenge inoculation with *R. solani* showed 4 isoforms PO1-PO4, but the control plant revealed only three isoforms and these isoforms were with less intensity (Plate 11, 12).

The infestation of the leaffolder in the mixture strain bacterized plants results in the induction of five isoforms PO1 to PO5. The PF1 treated plants showed three isoforms

Table. 13 Phenolic content in *Pseudomonas* treated rice plants inoculated with *R. solani* and leafroller

Treatments	Phenolic content* in <i>R. solani</i> inoculated plant (h)							Phenolic content* in leafroller inoculated plants (h)						
	0	24	48	72	96	120	168	0	24	48	72	96	120	168
PF1	237.50 ^{bc}	382.00 ^c	474.00 ^b	501.50 ^{ab}	395.00 ^b	390.50 ^b	373.50 ^{bc}	480.50 ^b	767.50 ^c	778.50 ^{cd}	593.50 ^e	545.50 ^{cd}	530.00 ^b	523.50 ^b
PF1 + Chitin	266.00 ^a	446.00 ^a	482.00 ^b	512.00 ^a	396.50 ^b	393.50 ^b	386.50 ^{abc}	486.00 ^{ab}	788.50 ^b	792.00 ^{bc}	608.50 ^e	558.50 ^{bc}	536.50 ^b	530.50 ^b
FP7	211.00 ^d	353.00 ^d	478.50 ^b	484.00 ^{bc}	388.50 ^b	387.00 ^b	363.00 ^c	480.50 ^b	756.50 ^c	763.50 ^d	760.00 ^e	532.50 ^d	504.00 ^c	482.50 ^c
FP7 + Chitin	214.50 ^{cd}	355.50 ^d	469.00 ^b	473.50 ^c	399.50 ^b	374.00 ^b	372.50 ^{bc}	485.50 ^{ab}	763.50 ^c	787.50 ^{bc}	787.50 ^b	535.00 ^d	508.50 ^c	485.50 ^c
PF1 + FP7	246.50 ^{ab}	412.00 ^b	514.50 ^a	505.00 ^{ab}	426.50 ^a	430.00 ^a	390.50 ^{ab}	492.00 ^{ab}	797.00 ^{ab}	805.50 ^b	810.50 ^a	570.00 ^{ab}	567.50 ^a	533.00 ^{ab}
PF1 + FP7 + Chitin	252.00 ^{ab}	425.00 ^{ab}	528.00 ^a	505.50 ^{ab}	435.00 ^a	435.00 ^a	401.50 ^a	509.50 ^a	813.00 ^a	834.00 ^a	830.50 ^a	580.50 ^a	575.50 ^a	552.00 ^a
Control	205.50 ^d	311.50 ^e	424.50 ^c	329.00 ^d	307.50 ^c	310.50 ^c	292.50 ^d	432.50 ^c	639.00 ^d	656.50 ^e	660.00 ^d	489.50 ^e	490.00 ^c	455.00 ^d

* ug of catechol/g of fresh tissue at 725 nm

Values are mean of two replications

Data followed by the same letter in a column are not significantly different from each other according to

Duncan's multiple range test at P= 0.05

CD for Treatment x hour (*R. solani*) : 12.08

CD for Treatment x hour (Leafroller) : 20.14

Table. 12 Effect of *P. fluorescens* on accumulation of lignin in sheaths and leaves of rice plant

Treatments	Inoculated with <i>R.solani</i> (%)	% increase over control	Inoculated with Leaf folder (%)	% increase over control
PF1	15.831 ^d	26.31	15.433 ^{de}	27.89
PF1 + Chitin	15.563 ^{cd}	12.42	15.198 ^d	25.94
FP7	15.590 ^{cd}	24.39	14.332 ^c	18.77
FP7 + Chitin	14.823 ^{bcd}	18.27	15.730 ^e	30.35
PF1+ FP7	17.299 ^e	38.02	17.000 ^f	40.8
PF1+ FP7+ Chitin	18.033 ^e	43.88	17.101 ^f	41.7
Control	12.533 ^a	-	12.067 ^a	-

Values are mean of two replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at P= 0.05

**Plate 6. SDS - PAGE analysis of crude protein extract of rice plants
inoculated with *R. solani***

Lanes

- 1. Marker**
- 2. Control + Pathogen**
- 3. Control**
- 4. Bacterized + Pathogen**
- 5. Bacterized**

Plate 6

**Plate 7. SDS - PAGE analysis of crude protein extract of rice plants
inoculated with *C. medinalis***

Lanes

- 1. Marker**
- 2. Control**
- 3. Control+Leaffolder**
- 4. Bacterized**
- 5. Bacterized+Leaffolder**

Plate 7

**Plate 8. SDS - PAGE analysis of crude protein extract of rice plants without
*C. medinalis***

Lanes

- 1. Marker**
- 2. Control**
- 3. PF1**
- 4. PF1+chitin**
- 5. FP7**
- 6. FP7+chitin**
- 7. PF1+FP7**
- 8. PF1+FP7+chitin**

Plate 8

Plate 9. SDS - PAGE analysis of crude protein extract of rice plants inoculated with *C. medinalis*

Lanes

- 1. Marker**
- 2. Control+Leaffolder**
- 3. PF1+Leaffolder**
- 4. PF1+chitin+Leaffolder**
- 5. FP7+Leaffolder**
- 6. FP7+chitin+Leaffolder**
- 7. PF1+FP7+Leaffolder**
- 8. PF1+FP7+chitin+Leaffolder**

Plate 9

Plate 10. SDS - PAGE analysis of crude protein extract of rice plants inoculated with and without *C. medinalis*

Lanes

- 1. Marker**
- 2. Control**
- 3. Control+Leaffolder**
- 4. PF1**
- 5. PF1+Leaffolder**
- 6. FP7**
- 7. FP7+Leaffolder**
- 8. PF1+FP7**
- 9. PF1+FP7+Leaffolder**

Plate 10

Plate 11. Native gel electrophoresis (8%) of peroxidase of sheaths from rice plants treated with PF1+FP7 strain at different hours after inoculation with *R. solani*

Control
Lanes

1. 0 h
2. 24 h
3. 48 h
4. 72 h
5. 96 h
6. 120 h

P. fluorescens
Lanes

8. 0 h
9. 24 h
10. 48 h
11. 72 h
12. 96 h
13. 120 h

Plate 12. Native gel electrophoresis (8%) of peroxidase of sheaths from rice plants treated with PF1 strain at different hours after inoculation with *R. solani*.

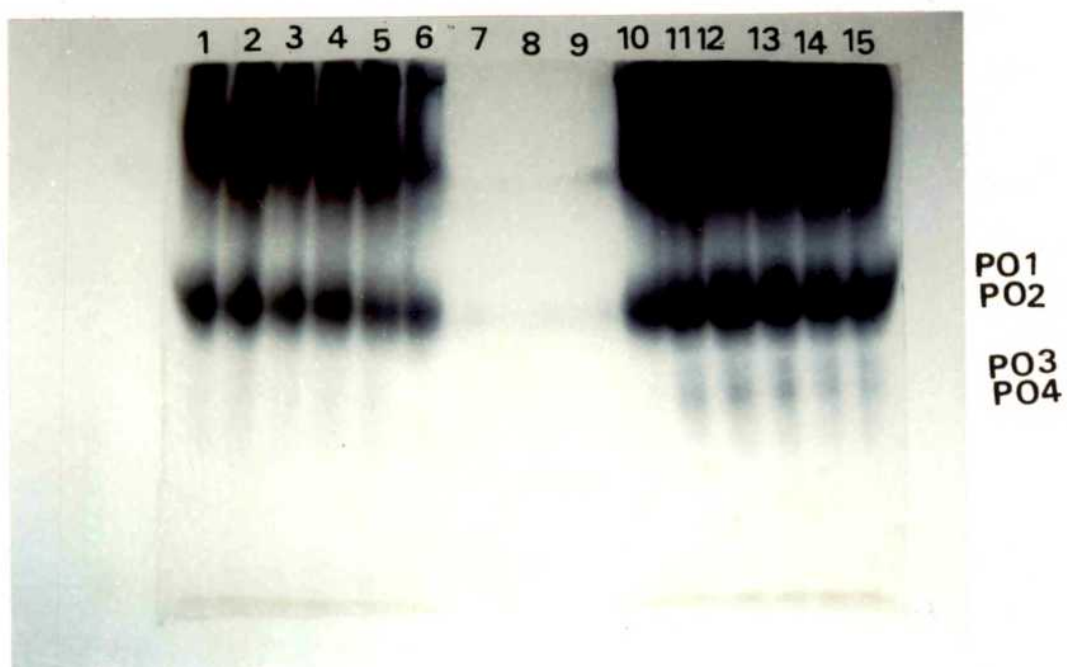
**Control
Lanes**

**1. 0 h
2. 24 h
3. 48 h
4. 72 h
5. 96 h
6. 120 h**

***P. fluorescens*
Lanes**

**10. 0 h
11. 24 h
12. 48 h
13. 72 h
14. 96 h
15. 120 h**

Plate 12



(PO1, PO2 and PO5) after challenging with leaffolder. However, in the FP7 treated plants there was no induction of peroxidase isoforms PO3, PO4 and PO5 (Plate 13).

4.8. Isoform pattern of polyphenol oxidase

Isoform pattern of the PPO in the rice sheath and leaf challenged with or without *R. solani* and *C. medinalis* respectively were studied. The PPO isozyme pattern of *Pseudomonas* treated tissues taken from 35 days old plants after challenge inoculation with pathogen revealed the presence of a unique isozyme PPO4 detected only in the bacterized plant and remains as dark band without change over time. But in the control plants the isozymes become faint immediately after 48 h of challenge inoculation

The unique enzyme band was not visible in gels of extracts prepared from leaf tissues of control plant. Native PAGE analysis of the PGPR untreated tissues after pathogen or leaffolder inoculation showed comparatively lesser induction of PPO isoforms. The intensity of the isoforms (PPO2 and PPO3) increased and became faint only after 120 h of leaffolder infestation in the bacterized plants. However, the isozymes appeared with higher intensity compared to the control plants after infestation. The PPO2 isoform is induced only in the PF1 treated and mixture treatment after leaffolder infestation (Plate 14, 15, 16, 17).

4.9. Isoform pattern of chitinase

Western blot detected two chitinase isoforms with molecular weight of 28 and 35 kDa with barley chitinase antiserum. In control plants inoculated with *R. solani* both these chitinases were detected but they were not well pronounced in intensity as compared to the *Pseudomonas* treated plants challenged with the pathogen. In uninoculated control plant, the constitutive 35 kDa chitinase was expressed, whereas in the uninoculated *Pseudomonas* treated plants both 28 kDa and constitutive 35 kDa chitinases were expressed to the intensity as that of control *R. solani* inoculated plants (Plate 18). In the *Pseudomonas* treated plant the intensity of the proteins increased with time (Plate 19).

Table: 14. Effect of single and mixture of *Pseudomonas fluorescens* strains (with and with out chitin) against sheath blight incidence in rice under field conditions.

Treatments	Per cent Disease Index (PDI)		Per cent disease reduction over control	
	Trail I	Trail II	Trail I	Trail II
Pfl	24.44 ^b (29.58)	19.26 ^{ab} (26.02)	54.00	55.17
Pfl+Chitin	22.22 ^{ab} (28.11)	18.51 ^{ab} (25.48)	58.18	56.91
FP7	25.92 ^b (30.59)	22.96 ^{ab} (28.63)	51.22	46.55
FP7+Chitin	23.15 ^{ab} (28.75)	19.26 ^{aab} (26.02)	56.43	55.16
Pfl+FP7	22.16 ^{ab} (28.11)	17.03 ^a (24.36)	58.30	60.36
Pfl +FP7 + Chitin	19.99 ^{ab} (26.54)	16.29 ^a (23.79)	62.38	62.08
Carbendazim	19.26 ^{ab} (26.02)	17.77 ^{ab} (24.91)	63.76	58.63
Chlorpyriphos	34.05 ^c (35.67)	25.55 ^b (29.82)	35.92	40.52
Carbendazim+Chlorpyriphos	17.03 ^a (24.36)	19.25 ^{ab} (25.97)	67.97	55.19
Control	53.14 ^d (46.83)	42.96 ^c (40.94)	-	-

The data in the parentheses are arcsine transformed values.

Values are mean of three replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

Plate 13. Native gel electrophoresis (8%) of Peroxidase of leaves from rice plants treated with *Pseudomonas* strain at different hours after inoculation with *C.medinalis*.

Lanes

- 1. Control+Leaffolder**
- 2. Control**
- 3. PF1+FP7+Leaffolder**
- 4. PF1+FP7**
- 5. PF1+Leaffolder**
- 6. PF1**
- 7. FP7+Leaffolder**
- 8. FP7**

Plate 13



Plate 14. Native gel electrophoresis (8%) of PPO of sheaths from rice plants treated with PF1+FP7 strain at different hours after inoculation with *R. solani*.

Control

Lanes

1. 0 h
2. 24 h
3. 48 h
4. 72 h
5. 96 h
6. 120 h

***P. fluorescens* (PF1+FP7)**

Lanes

8. 0 h
9. 24 h
10. 48 h
11. 72 h
12. 96 h
13. 120 h

Plate 14



PP01
PP02
PP03
PP04

Plate 15. Native gel electrophoresis (8%) of PPO of sheaths from rice plants treated with PF1 strain at different hours after inoculation with *R. solani*.

Control

Lanes

1. 0 h
2. 24 h
3. 48 h
4. 72 h
5. 96 h
6. 120 h

***P. fluorescens* (PF1)**

Lanes

8. 0 h
9. 24 h
10. 48 h
11. 72 h
12. 96 h
13. 120 h

Plate 15

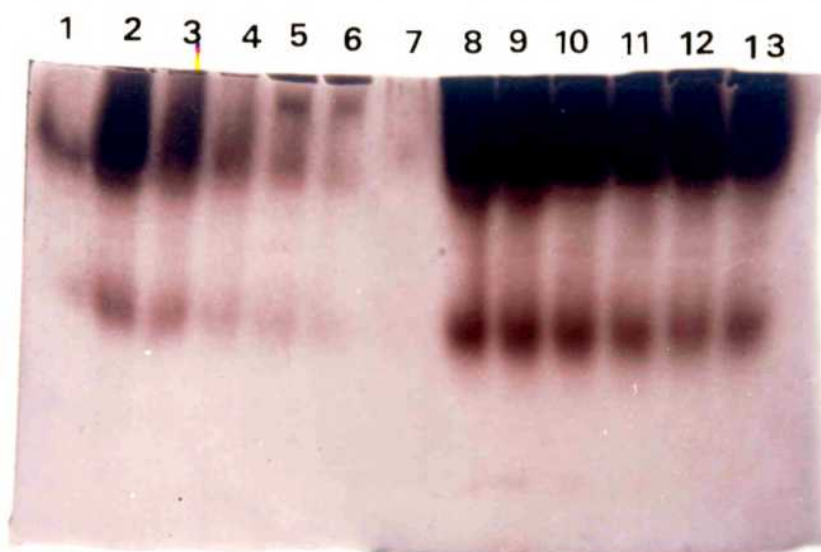


Plate 16. Native gel electrophoresis (8%) of PPO of rice leaves inoculated with *C.medinalis*.

Lanes

- 1. Control+Leaffolder**
- 2. Control**
- 3. PF1+FP7+ Leaffolder**
- 4. PF1+FP7**
- 5. FP7+ Leaffolder**
- 6. FP7**
- 7. PF1+ Leaffolder**
- 8. PF1**

Plate 16

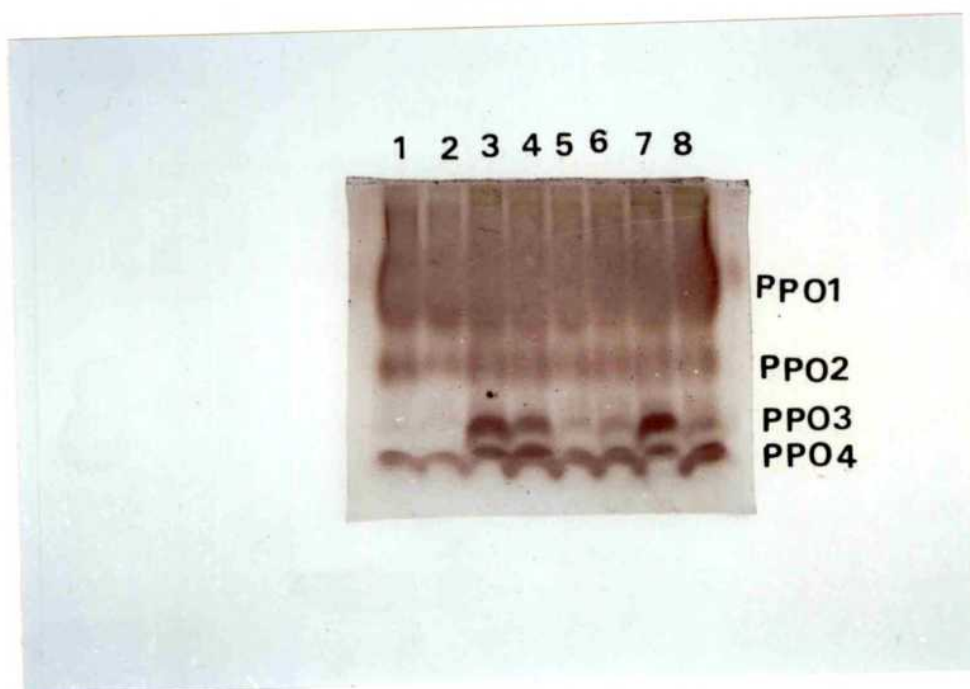


Plate 17. Native gel electrophoresis (8%) of PPO in rice leaves at different intervals after inoculation with *C. medinalis*

Control
Lanes

1. 0 h
2. 24 h
3. 48 h
4. 72 h
5. 96 h
6. 120 h

***P. fluorescens* (PF1+FP7)**
Lanes

8. 0 h
9. 24 h
10. 48 h
11. 72 h
12. 96 h
13. 120 h

Plate 17



Plate 18. Western blot analysis of crude protein extract probed with barley chitinase antiserum of rice sheath inoculated with *R. solani*

Lanes

- | | |
|-------------------|---------------------------------------|
| 1. Marker | 9. Control+ <i>R. solani</i> |
| 2. Control | 10. PF1+FP7+ <i>R. solani</i> |
| 3. PF1+FP7 | 11. PF1+FP7+chitin + <i>R. solani</i> |
| 4. PF1+FP7+chitin | 12. FP7+ <i>R. solani</i> |
| 5. FP7 | 13. FP7+chitin+ <i>R. solani</i> |
| 6. FP7+chitin | 14. PF1+ <i>R. solani</i> |
| 7. PF1 | 15. PF1+chitin+ <i>R. solani</i> |
| 8. PF1+chitin | |

Plate 18

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

kDa

35

28

Plate 19. Western blot analysis of crude protein extract probed with chitinase antiserum from rice sheath at different hours after inoculation with *R. solani* (in PF1+FP7 treated plants)

Lanes

- 1. Marker**
- 2. 0 h**
- 3. 24 h**
- 4. 48 h**
- 5. 72 h**
- 6. 96 h**
- 7. 120 h**

Plate 19

7 6 5 4 3 2 1

 κ Da

35

28

Western blot analysis of extract from leaf revealed the induction of the chitinase isoforms with molecular weight of 35 kDa and 56 kDa chitinases. In *Pseudomonas* treated plant after infestation with leaffolder increased levels of both chitinase isoforms were seen when compared to the infested control plants. Chitinase levels were increased in response to bacterization with *P. fluorescens* compared to healthy plants. The only significant difference in the responses between the treated and control was the intensity of the chitinase isoforms, which are not well pronounced in the control plant (Plate 20, 21).

4.10. Purification of chitinase

Chitinase present in *Pseudomonas* treated (PF1+FP7) plants were purified to apparent homogeneity by ammonium sulphate precipitation and chitin affinity chromatography. The chitinase of apparent molecular weight 35 kDa appear to be greater than 95 % pure by western blot detection with barley chitinase antiserum (Plate 22, 23).

4.11. Inhibition of fungal growth by purified chitinase

The effect of purified chitinase on the mycelial growth of phytopathogenic fungus *R. solani* was studied. The purified chitinase at 20 µg and crude extract at 40 µg show inhibition of the *R. solani* (Plate 24).

4.12. Effect of *Pseudomonas* treatment on sheath blight and leaffolder incidence under field conditions

Results from both the field trials indicated that the talc-based formulation of *Pseudomonas* strains either as individually or as mixture (with and without chitin) significantly suppressed the sheath blight and leaffolder incidence. The efficacy of *Pseudomonas* strain mixture application was equivalent or better than the standard fungicide application in controlling the disease severity in both field trials I and II. The effect of *P. fluorescens* on the sheath blight incidence and leaffolder infestation was examined and results are shown in table 14 and table 15 respectively. Significant reduction of total disease incidence was observed in the mixture treatment (62.4 and 62.0%) when compared with the control (53.1 and 42.9%) in trail I and II respectively.

Plate 20. Western blot analysis of crude protein extract of rice leaves probed with chitinase antiserum inoculated with *C. medinalis*.

Lanes

- 1. Marker**
- 2. Control**
- 3. Control+Leaffolder**
- 4. PF1**
- 5. PF1+Leaffolder**
- 6. FP7**
- 7. FP7+Leaffolder**

Plate 20

1 2 3 4 5 6 7

kDa

56

35

Plate 21. Western blot analysis of crude protein extract of rice leaves probed with chitinase antiserum inoculated with *C. medinalis*.

Lanes

- | | |
|-------------------|---|
| 1. Marker | 9. Control+ <i>C. medinalis</i> |
| 2. Control | 10. PF1 + <i>C. medinalis</i> |
| 3. PF1 | 11. PF1 +chitin + <i>C. medinalis</i> |
| 4. PF1+chitin | 12. FP7+ <i>C. medinalis</i> |
| 5. FP7 | 13. FP7+chitin+ <i>C. medinalis</i> |
| 6. FP7+chitin | 14. PF1+FP7+ <i>C. medinalis</i> |
| 7. PF1+FP7 | 15. PF1+FP7+chitin+ <i>C. medinalis</i> |
| 8. PF1+FP7+chitin | |

Plate 21

15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

kDa

56

35

Plate 22. Western blot analysis of purified chitinase from *Pseudomonas* treated rice sheath after inoculation with *R. solani*.

Lanes

- 1. Marker**
- 2. Purified chitinase**

Plate 23. SDS PAGE analysis of purified chitinase from *Pseudomonas* treated rice sheath after inoculation with *R. solani*.

Lanes

- 1. Marker**
- 2. Purified chitinase**

Plate 22

93

kDa

2

1

35

Plate 23

kDa

35

97

68

43

29

20

14

Plate 24. Antifungal activity of purified chitinase from *P. fluorescens* treated plant after challenged with *R. solani*.

Filter paper disc

- 1. Buffer**
- 2. Crude extract 40µg concentration**
- 3. Purified chitinase 20µg concentration**

Plate 24



However, in the case of leaffolder, maximum control (80.0 % and 73.3%) was achieved by application of chlorpyrifos in trial I and II respectively, followed by *Pseudomonas* combination treatments (47.7% and 56.1%) (Table 15). The leaffolder adult population under field condition was also significantly lower in *P. fluorescens* treated plants next to the chemical treatment (Table 16). The level of protection of rice plants from sheath blight and leaffolder was further slightly enhanced when chitin was added in the formulation (Plate 25, 26).

4.13. Effect of the *Pseudomonas fluorescens* on growth parameter under field conditions

Bacterized plants grew taller and appeared greener than did those in non bacterized field plots. At 35 DAP, the bacterized plants were on an average taller than the non bacterized plants (Table 17). The enhanced plant height and increased tiller number/hill were observed in the *P. fluorescens* treated plants. Among the treatments the mixture with chitin showed significantly increased number of tillers per hill and plant height. An average of 3.3 increased tillers per hill were noticed in plants treated with strain mixture amended with chitin than the control plot (Plate 27).

4.14. Effect of *Pseudomonas* treatment on grain yield under field conditions:

The effect of induced resistance on sheath blight and leaffolder and its consequence on grain yield was examined in *Pseudomonas* treated plants. The bacterized plants recorded higher yield than the untreated and fungicide treated plants in both the field trials I and II. The number of grains and weight of the grains was higher in the bacterized plants. Maximum of 12% and 21.1% increased yield was observed in two trials respectively (Table 18). The highest yield of 6.53 and 5.04 tonnes/ha was observed in plots bacterized with strain mixture + chitin as against the control plot which recorded only 5.8 and 3.84 tonnes/ha in trials I and II. The yield was also significantly greater than the untreated control when bacterized with individual strains PF1 (6.28 and 4.64) and FP7 (6.21 and 4.55).

Table: 15. Effect of single and mixture of *Pseudomonas fluorescens* strains against leaffolder infestation in rice under field conditions.

Treatments	Per cent leaffolder incidence		Per cent leaffolder infestation reduction over control	
	Trail I	Trail II	Trail I	Trail II
Pfl	15.03 ^{cd} (22.81)	12.62 ^{de} (20.79)	30.48	29.92
Pfl+Chitin	13.46 ^c (21.52)	11.44 ^{de} (19.76)	37.74	36.48
FP7	15.88 ^d (23.48)	13.46 ^c (21.50)	26.54	24.48
FP7+Chitin	14.93 ^{cd} (22.70)	10.7 ^{cd} (19.06)	30.94	40.58
Pfl+FP7	11.6 ^b (19.91)	9.41 ^{bc} (17.86)	46.34	47.75
Pfl+FP7 + Chitin	11.26 ^b (19.59)	7.93 ^b (16.34)	47.91	55.96
Carbendazim	20.74 ^e (27.09)	13.54 ^e (21.58)	4.70	24.81
Chlorpyriphos	4.25 ^a (11.89)	4.83 ^a (12.65)	80.34	73.18
Carbendazim+ Chlorpyriphos	5.21 ^a (13.16)	3.99 ^a (11.52)	75.90	77.84
Control	21.62 ^e (27.70)	18.01 ^f (25.11))	-	-

The data in the parentheses are arcsine transformed values.

Values are mean of three replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

Table: 16. Effects of *Pseudomonas* on Leaffolder adults population under field condition

Treatments	No. of leaffolder adults/sq m
Pfl	9.00 ^{cde}
Pfl+Chitin	8.00 ^{bcd}
FP7	9.00 ^{cde}
FP7+Chitin	10.00 ^{de}
Pfl+FP7	7.33 ^{abc}
Pfl+FP7 + Chitin	6.67 ^{abc}
Carbendazim	11.00 ^e
Chlorpyriphos	6.00 ^{ab}
Carbendazim+ Chlorpyriphos	5.00 ^a
Control	13.67 ^f

Values are mean of three replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

Table: 17. Effect of *Pseudomonas fluorescens* on tiller number and plant height under field conditions:

Treatments	Tiller number/hill		Plant height in cm	
	Trail I	Trail II	Trail I	Trail II
Pfl	10.20 ^c (21.47)	10.87 ^{bc} (25.37)	73.05 ^c (12.29)	70.71 ^b (10.43)
Pfl+Chitin	10.27 ^c (22.26)	12.10 ^c (39.56)	73.74 ^c (13.36)	71.33 ^b (11.40)
FP7	8.60 ^{ab} (2.38)	10.47 ^{bc} (20.76)	70.83 ^d (8.88)	68.33 ^{cd} (6.71)
FP7+Chitin	10.267 ^c (22.26)	11.40 ^{bc} (31.48)	71.47 ^d (9.87)	69.30 ^c (8.23)
Pfl+FP7	13.20 ^f (57.14)	13.87 ^d (59.97)	75.91 ^b (16.69)	73.70 ^a (15.10)
Pfl+FP7 + Chitin	13.67 ^f (62.38)	14.00 ^d (61.47)	77.09 ^a (18.51)	74.57 ^a (16.46)
Carbendazim	9.067 ^{bc} (7.94)	8.80 ^a (1.49)	69.40 ^e (6.69)	67.17 ^{ef} (4.91)
Chlorpyriphos	9.27 ^{cd} (10.35)	9.73 ^{ab} (12.23)	66.87 ^f (2.79)	66.57 ^f (4.44)
Carbendazim+Chlorpyriphos	9.80 ^{de} (16.66)	9.93 ^{ab} (14.54)	69.35 ^e (6.61)	67.87 ^{de} (5.99)
Control	8.40 ^a	8.67 ^a	65.05 ^g	64.03 ^g

Values are mean of three replications

Values in the parentheses are per cent increase over control

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

Table: 18. Effect of single and mixture of *Pseudomonas fluorescens* strains on grain yield in rice under field conditions.

Treatments	Yield tonnes/ha ^z		Percent increase over control	
	Trail I	Trail II	Trail I	Trail II
Pfl	6.28 ^{cd}	4.64 ^{cd}	8.27	22.11
Pfl+Chitin	6.32 ^{de}	4.80 ^d	8.96	26.32
FP7	6.21 ^{cd}	4.55 ^c	7.06	19.74
FP7+Chitin	6.33 ^{de}	4.59 ^c	9.14	20.79
Pfl+FP7	6.40 ^e	4.80 ^d	10.34	26.32
Pfl+FP7 + Chitin	6.53 ^f	5.04 ^e	12.58	32.63
Carbendazim	5.98 ^b	4.36 ^b	3.10	14.73
Chlorpyriphos	6.00 ^b	4.67 ^{cd}	3.45	22.89
Carbendazim+ Chlorpyriphos	6.19 ^c	4.29 ^b	6.72	12.89
Control	5.80 ^a	3.84 ^a	-	-

Values are mean of three replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

^zThe grain yield was weighed for 20 m² and calculated for one hectare.

Plate 25. Field view of *P. fluorescens* strains applied plots (Trial I and Trial II)

25a Pondicherry (Trial I)

25b Coimbatore (Trail II)

Plate 26. Effect of *P. fluorescens* formulation on rice sheath blight incidence under field condition

- a. *P. fluorescens* treated**
- b. control**

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Plate 26a



Plate 26b



Plate 27 Effect of *P. fluorescens* formulation on plant growth under field condition

- a. *P. fluorescens* treated**
- b. control**

Plate 27a



Plate 27b



Yield in the mixture treatment recorded 5.9 and 7.5% increased yield than the individual strains PF1 and FP7 respectively. The amendment of chitin to the *P. fluorescens* formulation was 3.1 % greater than the formulation without the chitin, although differences were not significant ($P = 0.05$).

4.15. Occurrence of natural enemies

It was interesting to note that, the occurrence of natural enemy (hymenopterous parasitoid and spider) population increased in *Pseudomonas* treated plants as compared to chemical and control treatments. Invariably in all the treated plants, an average of nine hymenopterans was collected per four sweeps as against 1 to 2 in chemical treatment. Likewise, the spider population was also increased (4-7 per 10 hill) as against (3-4 per hill) in chemical treatment (Table 19, Plate 28).

4.16. Bacterial growth in the modified medium

The growth of bacterial strains varied in the media amended with chitin from the media without chitin. The bacterial growth was significantly higher in the chitin amended King's B medium. The population of the FP7 was significantly higher than PF1 strain when inoculated individually without chitin. The medium inoculated with both strains recorded significantly higher population. However, in the medium amended with chitin also increased the bacterial population (Table 20).

4.17. Chitinase Production by bacterial strains in the culture medium

The cultural filtrates of the bacterial strains were used to assess the chitinase activity. Chitinase activity was higher in the PF1+FP7 strains inoculated medium amended with chitin. Among individual strains of PF1 and FP7, FP7 recorded significantly higher chitinase activity (Table 21). The results from the table revealed that chitin amendment increased the chitinase activity of the bacterial strains.

Table: 19. Natural enemies population of leaffolder in field (Trail II)

Treatments	No. of hymenopteran parasitoids / 5 sweeps	No. of Predacious spider population / 10 hills	
		45 th day	55 th day
Pfl	7.33 ^{bc}	5.33 ^{bc}	7.33 ^{cde}
Pfl+Chitin	8.00 ^{bc}	6.00 ^{cd}	8.33 ^{de}
FP7	6.67 ^b	4.33 ^{ab}	5.33 ^{abc}
FP7+Chitin	7.67 ^{bc}	5.33 ^{bc}	6.33 ^{bcd}
Pfl+FP7	9.00 ^c	6.33 ^{cd}	9.33 ^e
Pfl+FP7 + Chitin	9.33 ^c	7.00 ^d	9.33 ^e
Carbendazim	3.33 ^a	4.33 ^{ab}	5.33 ^{abc}
Chlorpyriphos	2.00 ^a	3.67 ^a	4.33 ^{ab}
Carbendazim+ Chlorpyriphos	1.67 ^a	3.00 ^a	3.67 ^a
Control	6.00 ^b	4.00 ^{ab}	6.67 ^{cd}

Values are mean of three replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

Table 20. Population of bacterial strains and its mixture in medium amended with or with out chitin

Treatments	Bacterial population $\times 10^8$ cfu
King's B medium:	
PF1	1.6501 ^b
chitin+PF1	1.9575 ^c
FP7	2.2864 ^d
Chitin+FP7	2.5987 ^e
PF1+FP7	2.3367 ^d
Chitin+PF1+FP7	3.2201 ^f

Values are mean of three replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$

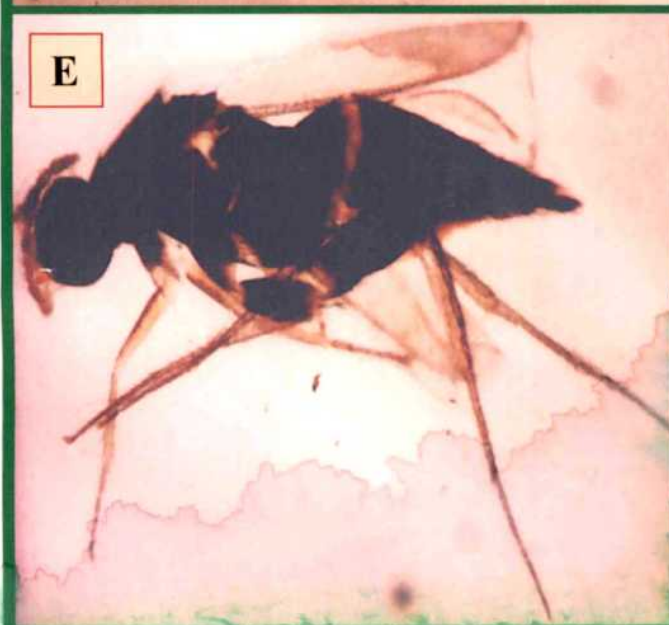
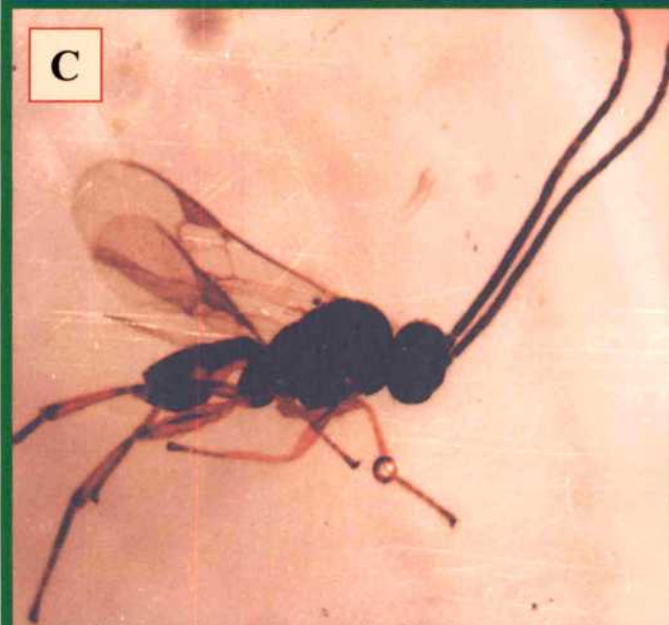
Table: 21. Chitinase activity in culture filtrates of bacterial strains grown individually and in mixture

Treatment	Chitinase activity*
Pf1	0.0370 ^a
Pf1+Chitin	0.0410 ^b
FP7	0.0470 ^c
FP7+Chitin	0.0483 ^c
Pf1+FP7	0.0533 ^d
Pf1+FP7 + Chitin	0.0557 ^d

* $\mu\text{mol of GlcNAc min}^{-1} \text{ ml}^{-1}$

Values are mean of three replications

Data followed by the same letter in a column are not significantly different from each other according to Duncan's multiple range test at $P = 0.05$



Discussion

CHAPTER V

DISCUSSION

Leaffolder and sheath blight are the major insect pest and disease respectively that occur wherever rice is cultivated. Researchers worldwide have focussed their efforts on understanding and managing the major diseases and pests through traditional, cultural, biological, chemical and more recently biotechnological approaches. Though each and every method has its own merits and demerits, pest and disease management through biological approach is now gaining importance for the reason, it is more reliable and safe alternative to conventional chemical methods of control and ecologically as well as economically sustainable.

The development of biological techniques using *Pseudomonas* strains is an emerging field in crop protection to reduce the damage of economic crop caused by plant pathogens and insects. In spite of major advances in understanding the plant defense response, little information is available on rhizobacteria mediated induced resistance against the insect pest. However most of the research studies involving biocontrol have been performed in the laboratory and greenhouse or under less than natural conditions with cultured fungi and bacteria. In recent years, the interest among the regulatory authorities, agricultural advisors and growers changed to use microbial products as agents for pest and disease management. Many strains of *P. fluorescens* 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 pest and diseases (Nandakumar *et al.*, 2000; Viswanathan *et al.*, 2000; Qingwen *et al.*, 1998; Zehnder *et al.*, 1997a; Rabindran and Vidhyasekaran, 1996; Rosales *et al.*, 1993). This has resulted in the commercialization of a number of microbial biocontrol products (Fravel and Larkin, 1996). Hence, talc-based formulation containing single and mixture of *Pseudomonas* strains amended with or without chitin were tested against both sheath blight and leaffolder incidence under glass house and field conditions.

The applications of talc-based formulation through seed, root, foliar and soil significantly reduced the sheath blight and leaffolder incidence. The efficiency by this bacterial strain mixture in disease suppression was comparable with fungicide carbendazim. *Pseudomonas* treated plants showed smaller sheath blight lesions and reduced leaf area damage compared to the untreated plants upon inoculation. The leaffolder infestation was also less in the *Pseudomonas* strain mixture treatments than the control plants though the infestation was high when compared with the chlorpyrifos treatment. Upon challenge inoculation with *C. medinalis* in *Pseudomonas* treated plants, the per cent leaf area damage was less compared to the untreated plants.

Seed bacterization of rice with bacterial strains checked the development of sheath blight symptoms (Rosales *et al.*, 1993; Vasanthadevi *et al.*, 1989; Mew and Rosales, 1986). Further, the combined application of fluorescent pseudomonads as root dip, foliar and soil application along with seed treatment enhanced sheath blight disease protection both under greenhouse and field conditions (Nandakumar *et al.*, 2000; Vidhyasekaran and Muthamilan, 1999; Rabindran and Vidhyasekaran, 1996). Seed treatment followed by foliar application of fluorescent pseudomonads at 30, 45, 60 days after planting controlled sheath and blast disease of rice and increased the yield (Chatterjee *et al.*, 1996). Few workers have also reported the use of strain mixture in disease management. Application of *P. fluorescens* strain mixture of 2-79 and 13-79 reduced the take-all disease intensity in wheat compared to 13-79 alone in six field trials (Weller and Cook, 1983), two to four strain mixture gave better control of take-all than individual strains (Pierson and Weller, 1994) and mixture of strain PF1 and FP7 was found to reduce the sheath blight disease incidence under field condition (Nandakumar *et al.*, 2000). The management of insect pest, plant diseases and nematodes by different *Pseudomonas* strains either as bacterial suspension or through different formulations have been reported by many workers (Viswanathan and Samiyappan, 1999; Swarnakumari *et al.*, 1999; Krishnamurthy and Gnanamanickam, 1998; Zehnder *et al.*, 1997a; Vidhyasekaran *et al.*, 1997a; b; 1999; Raupach *et al.*, 1996; Liu *et al.*, 1995a; Santhi and

Sivakumar, 1995; Maurhofer *et al.*, 1994; Racke and Sikora, 1992; Sikora, 1992, 103 Kloepper and Schroth, 1982).

Numerous mode of action have been postulated and demonstrated for antagonistic effects of *Pseudomonas fluorescens* in controlling soil-borne diseases which includes production of secondary metabolites and antifungal compounds (Dunne *et al.*, 1998; Dowling and O'Gara, 1994), competition for nutrients (O'Sullivan and O'Gara, 1992), production of cell wall lytic enzymes (chitinase and glucanase) that act on fungal cell wall components (Singh *et al.*, 1999) and more importantly through induced systemic resistance (ISR). Further several studies indicated that the PGPR strains may stimulate the production of biochemical compounds associated with host defense (M'Piga *et al.*, 1997; Zdor and Anderson, 1992). Moreover the salicylic acid produced and the lipopolysaccharide of *P. fluorescens* acts as local and systemic signal molecules in inducing resistance in plants (De Meyer and Hofte, 1997; Van Peer and Schippers, 1992). The strains used in the present study, PF1 and FP7 were found to induce more amount of chitinase and peroxidase, which are the most important components of induced systemic resistance (ISR) (Nandakumar *et al.*, 2000; Dalisay and Kuc, 1995). The possible mechanisms of insect control by *P. fluorescens* are unknown. The data of the present study indicate that the *Pseudomonas* treatment influenced the growth and development of insects at all stages of growth (larva, pupa and adult). *P. fluorescens* greatly affected the feeding behaviour of the larvae resulting in reduced larval and pupal weight, increased larval mortality and malformed adult emergence. *Helicoverpa zea* larvae, the corn earworm reared on bacteria (*Pseudomonas maltophilia*) contaminated diet adversely affected the larval growth resulting in more than 60 per cent reduction in adult emergence (Bong and Sikorowski, 1991). Pupae and adults emerging from these bacteria-infected larvae remained smaller. Yaman *et al.* (1999) reported the insecticidal effect of *P. fluorescens* isolated from sea water against chestnut weevil, most damaging pest of chestnut and oak. The spraying of *P. fluorescens* suspension resulted in 20 per cent mortality of the weevil.

Plants possess active defense mechanism against pathogen attack when these defense mechanisms are activated by a stimulus prior to the infestation of the pest and the infection by the pathogen. Induced resistance with enhanced defensive capacity helps the plant to prevent the attack of insect pest and pathogen. The stimulus for enhanced defensive capacity to the plants is due to the biotic or abiotic inducers that can activate defense genes. The early and increased expression of these genes can induce systemic resistance in plants against various insects and pathogens in many crop plants including soybean, tomato, potato, wheat, cotton cucumber and rice (Chen *et al.*, 2000; Nandakumar *et al.*, 2000; Qingwen *et al.*, 1998; Karban and Baldwin, 1997; Bleichert *et al.*, 1995; Kogen and Paxton, 1983). Pre inoculation of plants with pathogen and insect exhibit increased production of reactive oxygen species, salicylic acid, ethylene and jasmonates (Yang *et al.*, 1997; Hammond-kosack and Jones, 1996). These molecules serve as secondary signals to activate the expression of PAL, peroxidase, phenols, PPO and PR genes. The biological function of PR proteins has remained mysterious for 15 years, but extensive investigation in the past decade has demonstrated the antimicrobial capabilities and antinutritional quality of this class of defense proteins.

A separate signaling pathway involving jasmonic acid (JA) is involved in systemic responses to wounding and insect herbivory. The signaling pathway of SAR and the octadecanoid pathway (Doares *et al.*, 1995), which is involved in the induced resistance against pathogen and herbivores may interact. Certain pathogens induce both SA and JA-dependent pathways (Pieterse *et al.*, 1999; Kenton *et al.*, 1999; Petrovic *et al.*, 1997). Likewise, aphid, whitefly and leafminer infestations elicit SA-dependent PR gene and protein expression without inducing JA-pathways (Inbar *et al.*, 1999; Petrovic *et al.*, 1997). Jasmonic acid, a central signal molecule of the octadecanoid pathway is produced within the few minutes after insect feeding (Bleichert *et al.* 1995) and it leads to the activation of several defense genes (Doares *et al.*, 1995). Proteinase inhibitors (PIs) are highly stable defensive proteins of plant tissues that are both developmentally regulated and induced in response to insect and pathogen (Koiwa *et al.*, 1997; Ryan, 1990). It was found that jasmonic acid and volatile derivative, methyl jasmonate were powerful inducers of Proteinase Inhibitor (PI) genes through octadecanoid pathway.

There are ample evidences to show that wound-signaling pathways sometime function independently of jasmonate and likewise, there are salicylate-independent responses to pathogens (Pieterse and Van Loon, 1999). Cross-resistance between herbivores and pathogens was reported in several crops (Kahl *et al.*, 2000; Stout *et al.*, 1999). Feeding of tomato by *H. zea* results in induction of systemic resistance to both *H. zea* and *P. syringae*. Further, inoculation of leaves with *Pseudomonas syringae* pv. *tomato* induced systemic accumulation of transcripts for protease inhibitors and pathogenesis-related proteins and induced systemic resistance to both *P. syringae* and *H. zea* (Stout *et al.*, 1999). Also, infection of field grown cucumber with the scab fungus *Cladosporium cucumerinum* had no effect on the herbivore densities of cucumber beetles or melon aphids feeding on it (Moran, 1998).

The role of oral factors in triggering antiherbivore defenses in response to insect has been recently reported, however pathogen derived elicitors are well characterized, compared to insect derived elicitor of plant defenses. The best-characterized insect elicitors are those isolated from Lepidopteran oral secretions that elicit a systemic release of volatile plant compounds (Pare and Tumlinson, 1999). Oral secretions from *M. sexta* were shown to increase ethylene and JA production with a concomitant suppression of nicotine but an increase in the production of volatile terpenoids (Kahl *et al.*, 2000). Saliva from noctuid caterpillar species such as *H. zea* contains high concentration of a glucose oxidase (Eichenseer *et al.*, 1999). The salivary glucose oxidase from *H. zea* elicited induced resistance to *H. zea* and *P. syringae* pv. *tomato* by accumulation of pathogenesis-related proteins (Stout *et al.*, 1999).

The plant defense-related enzymes including pathogenesis-related proteins (PR-proteins) are reported to accumulate in plants with the onset of induced systemic resistance. Fluorescent pseudomonads have been reported to induce systemic resistance in several crops by activating defense related genes (Chen *et al.*, 2000; Nandakumar *et al.*, 2000; Xue *et al.*, 1998; Dalisay and Kuc, 1995; Schneider and Uldrich, 1994; Maurhofer *et al.*, 1994; Zdor and Anderson, 1992) The treatment with various biotic and abiotic inducers led to an increase in activities of chitinase, β -1,3-glucanase, peroxidase,

polyphenol oxidases and phenylalanine ammonia lyase in cucumber and tobacco leaf¹¹² tissues however, the extent and time course of increase varied according to the inducer and host plant (Schneider and Uldrich, 1994)

In our study, *P. fluorescens* strains, PF1 and FP7 induced various defense-related proteins viz., chitinases, peroxidase, PPO, PAL and synthesis of phenolics and lignin against *R. solani* and *C. medinalis*. In general, the pathogen and leafhopper stimulated more enzyme accumulation than the PGPR alone in sheath and leaves of rice plants. Zdor and Anderson (1992) reported that colonization of bean root by fluorescent bacteria was correlated with induction of PR proteins. Maurhofer *et al.* (1994) reported that ISR by fluorescent pseudomonads was associated with accumulation of β -1,3-glucanase and chitinases. These defense enzymes did not accumulate in the control plant indicating involvement of PR proteins in induction of systemic resistance.

In the *Pseudomonas* treated plants, the barley chitinase antiserum detected two isoforms with molecular weights of 28 and 35 kDa in rice sheath and 35 and 56 kDa in rice leaves inoculated with *R. solani* and *C. medinalis* respectively. The two isoforms of rice sheath and leaves were also expressed constitutively in *Pseudomonas* treated plants and untreated plants. However its activity was more pronounced in pathogen and pest challenge inoculated plants. The available published information on bacteria mediated insect and disease control through the bacteria induced plant chitinases and bacterial chitinase would have played an important role by hydrolyzing the chitin as chitin constitute a structural component of gut linings of the insect and fungal cell wall. In the present study, the purified chitinase, 35 kDa from the mixture treatment (PF1+FP7) inhibited the growth of the *R. solani* under *in vitro*. The molecular weight of this chitinase is very close to that of rice bran chitinase (Anuratha *et al.*, 1992) and elicitor inducible antifungal chitinase from suspension-cultured rice cells (Velazhahan *et al.*, 2000). Chitin metabolism is an excellent target for selective pest control (Krammer and Muthukrishnan, 1997). Incorporation of purified chitinase preparation in the insect diet significantly reduced the growth and survival of several insects such as *Spodoptera* (Sampson and Gooday, 1998; Regev *et al.*, 1997), *Trichoplusia ni* (Broadway *et al.*,

1998) and midge (Sampson and Gooday, 1998). Further the transgenic tobacco plants expressing chitinase enhanced resistance to some lepidopteran pest (Ding *et al.*, 1997).

Increased peroxidase (PO) has been observed in a number of resistant interaction involving plant pathogenic fungi, bacteria and virus (Chen *et al.*, 2000; Nandakumar *et al.*, 2000; Dalisay and Kuc, 1995). Peroxidases have been implicated in a number of physiological functions that may contribute to resistance including oxidation of hydroxy cinnamyl alcohol into free radical intermediates (Gross, 1980), phenol oxidation (Schmid and Feucht, 1980), polysaccharide cross linking (Fry, 1986), cross-linking of extensin monomers (Everdeen *et al.*, 1988) and lignification (Walter, 1992) and also correlated with deposition of phenolic material into plant cell walls during resistant interaction (Graham and Graham, 1991).

Accumulation of peroxidase has been correlated with induced systemic resistance (ISR) in several plants (Chen *et al.*, 2000; Dalisay and Kuc 1995; Hammerschmidt *et al.*, 1982). Although peroxidase has little effect on insects (Dowd and Vega, 1996; Rahbe and Febvay, 1993), there is some evidence that peroxidase can contribute to insect resistance. Oxidation products of several corn allelochemicals produced by peroxidases were more toxic than precursor when produced in sucrose solution fed to the maize leaf-hopper, *Dalbulus maidis* (Dowd and Vega, 1996). The accumulation of allelochemical, cucurbitacin in PGPR treated cucumber plants has been attributed to have resistance against cucumber beetle (Zehnder *et al.*, 1997a). Induction of peroxidases in tomato may depend on type of insect and damage (Stout *et al.*, 1994). Enhanced peroxidase isozyme activity was associated with the hypersensitivity response of *Solanum dulcamara* to mites (Bronner *et al.*, 1991). Similarly, the increased polyphenol and terpenoid content in *Pseudomonas gladioli* treated cotton plants reduced the relative growth rate, consumption rate and digestibility of feed by larvae of *Helicoverpa armigera* (Qingwen *et al.*, 1998). In the biosynthesis of lignin and hydrogen peroxide, plant PO has been reported to catalyze the last step (Bruce and West, 1989). In the present study, the total of four isozymes (PO1-PO4) and five (PO1-PO5) were detected in strain mixture treated plant challenge inoculated with *R. solani* and *C. medinalis* respectively. PO was found to

accumulate with concomitant increase in the lignin in plants treated with *P. flourescens* following pathogen infection and insect infestation. The highest level of peroxidase activity occurred in leaffolder challenged bacterized plants with mixture of PF1 and FP7 stain and increased rapidly within 24 h period.

Peroxidase reduces H_2O_2 to water at the expense of electron from coniferyl alcohol and the process initiates the lignification chain reaction in plants (Smith *et al.*, 1991). It has been established that lignin depositions from barriers limit fungal penetration. The early and increased expression of the peroxidase enzymes would have involved in the biochemical reaction necessary for lignification which protected the plants from *R. solani* and *C. medinalis*.

The present experiment demonstrated that the application of *P. flourescens* induced the polyphenol oxidase (PPO) activity in both leaves and sheaths of the rice plants. The unique *Pseudomonas* induced PPO2 isoform was not visible in gels of extracts prepared from leaf tissues of control plant. The intensity of isoforms (PPO2 and PPO3) increased after 24 h and become faint only after 120 h of leaffolder infestation in the bacterized plants and these isozymes appeared with higher intensity compared to the control plants after infestation. The PPO2 isoform is induced only in the PF1 treated and mixture treated plants after leaffolder infestation. The PGPR untreated tissues after pathogen or leaffolder inoculation showed comparatively lesser induction of PPO isoforms. PPO activity contributed to the decrease of nutritional quality of attacked plants by converting soluble phenolic compounds to quinones that eventually prevent the digestion of proteins. Considerable evidence implicates both PPO and phenols in the inducible resistance of tomato foliage to lepidopteran larvae (Felton *et al.*, 1992).

The present study also indicated that treatment with *P. flourescens* induced the PAL activity and caused increased accumulation of the phenol. Maximum accumulation of phenolics occurred by 24 h after infestation of leaffolder and 72 h after infection by the sheath blight, concomitant with a marked increase of PAL activity. Phenolic compounds accumulated by plants are formed through phenylpropanoid metabolism.

Phenylpropanoid metabolism starts with the conversion of L-phenylalanine into *trans* cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL) (Massala *et al.*, 1980). Conversely, transgenic tobacco expressing varying levels of PAL were used to show that levels of induced resistance to pathogens and insects in the same plant lines can be inversely correlated (Howles *et al.*, 1996). The given PGPR strain-host interaction might have involved any of these mechanisms individually or more than one of them acting simultaneously to suppress sheath blight pathogen growth and development of different stages of leaf folder.

The *Pseudomonas* strains with ISR activity may increase the possibility of achieving a better pest and disease suppression compared to any other mechanisms of biocontrol (Raupach and Kloepper, 1998; Zehnder *et al.*, 1997a). Invariably in all the experiments of this study, strain mixture performed better than their respective single strain. So the designing of combination of strains as biocontrol agents, making use of multiple antifungal and anti insecticidal traits exhibited by them proved to be more advantageous. It ensures that at least one of the biocontrol mechanisms will be functional under the unpredictable field conditions faced by the released PGPR strains (Duffey *et al.*, 1996; Pierson and Weller, 1994).

The production and secretion of chitinase by non-pathogenic microorganisms may be important in the biological control of plant pathogenic fungi (Viswanathan and Samiyappan, 2000; Potgieter and Alexander, 1996; Sneh *et al.*, 1985). Bacteria are known to produce chitinases in culture medium. The chitinase producing ability could be increased by adding chitin or its oligomers as carbon source (Elad *et al.*, 1982). *S. marcescens* chitinases in *Escherichia coli* and *Pseudomonas* spp. effectively inhibited the mycelial growth of *S. rolfsii* and *Fusarium oxysporum* f. sp. *redolens* (Shapira *et al.*, 1989). Expression of *Manduca* chitinase in *P. fluorescens* inhibited the growth of *R. solani* (Samiyappan *et al.*, 1993). Here in this study addition of chitin in the King's B medium increased chitinase activity and the population of the antagonistic bacteria.

In the present study, the chitin amendment considerably increased the biocontrol activity of *Pseudomonas* against sheath blight and leaf folder. The chitin amendment has been used to improve the biocontrol activity of PGPR under the assumption that the number and activity of chitinolytic microorganisms capable of attacking the chitinous hyphal wall of pathogen will increase in response to chitin amendment. Kokalis-Burelle *et al.* (1991) demonstrated that the insoluble polymer chitin could selectively enhance the growth and antagonism of chitinolytic bacteria. The effect of chitin in inducing systemic resistance either applied alone or in combination with biocontrol agents were reported in tomato (Benhamou *et al.*, 1998) and in cucumber (El-Ghamou *et al.*, 1994). Ellis *et al.* (1998) reported that chitin amendment drastically reduced number of both stubby root nematodes (*Trichodorus* spp.) and Collembola (*Protaphorura* spp., millipedes, *Brachydesmus* spp. and wireworm, *Agriotes* spp.). The published information on chitin in insect control revealed that, the peritrophin-A domains within the chitinases from *Bombyx mori* and *Brugia malayi* have been implicated in binding chitin (Koga *et al.*, 1997; Venegas *et al.*, 1996). Analogous but unrelated cysteine-rich domains in other chitinases also have been implicated in chitin binding activity (Yamagami and Funatsu, 1996; Beintema, 1994; Iseli *et al.*, 1993; Flach *et al.*, 1992). It is likely therefore, that the peritrophin-A domain binds to chitin and justifies the active site of the chitinases. Another more active role of this domain could involve the disruption of hydrogen bonds involved in the stabilization of chitin fibrils or chitin-protein complexes thereby facilitating the hydrolytic process.

Laboratory studies indicated both root and shoot growth was promoted by treating seeds with *P. fluorescens*. This growth promotion confirms reports of Whipps and Lumsden (1991); Kaiser *et al.*, (1989). Though chemical treatment reduced the disease and insect incidence, *Pseudomonas* treatment played a dual role by protecting the rice plants and promoting the growth of the plant, which has ultimately led to increased biomass and yield. The *Pseudomonas* strain PF1 and FP7 either single or in combination promoted the plant growth and increased the grain yield (Nandakumar *et al.*, 2000; Vidhyasekaran *et al.*, 1999; 1997). There are several ways in which different PGPR have

been reported to directly facilitate the proliferation of their plant hosts. PGPR can synthesize siderophores that can solubilize and sequester iron from the soil and provide it to plant cells. They synthesize several phytohormones such as auxin, gibberellin and cytokinin that can act to various stages of plant growth and may have mechanisms for the solubilization of minerals such as phosphorus that become more readily available for the plant growth and may synthesize some less well characterized low molecular mass compounds or enzymes that can modulate plant growth and development (Glick *et al.*, 1994, Dubeikovsky *et al.*, 1993; Kloepper, 1980). A particular PGPR strain may effect plant growth and development by using any one or more of these mechanisms.

Rice leaffolder management can be improved when the effect of natural enemy densities on leaffolder infestations is known. Natural enemy conservation measures could be taken to sustain natural control (Shepard and Ooi, 1991). The increase in hymenopterous parasitoid and spider population in *Pseudomonas* treated plants may partly be responsible for the reduced incidence of leaffolder under field conditions. However, different approaches were needed to answer the question whether and which natural enemy can keep rice leaffolder populations below ETL and it will also be necessary to identify the major species within the large complex of leaf folder natural enemies. The increased natural enemy population might have associated with some host-PGPR based pathways (octadecanoid pathway) leading to the production of volatile compounds, which attracts the natural enemies. It has been suggested that jasmonic acid, which is produced from linolenic acid by the octadecanoid signaling pathway is a key regulatory component in the transduction sequence that triggers synthesis and release of volatile compounds by plants (Anurag, 2000; Krumm *et al.*, 1995).

The tropical rice fields harbour a large complex of natural enemies, that may keep rice leaf folder populations below damaging levels most of the time (Bellows *et al.*, 1992; Barrion *et al.*, 1991; Van Driesche, 1983). Effects of induced responses on natural enemies may also act additively or synergistically as is evident from the prolonged development of PGPR-induced allelochemicals, thereby attracting natural enemies which have evolved the capability to learn to use induced responses for host location. Besides

the release of volatile allelochemicals may further increase natural enemy activity. Leaves normally release small quantities of volatile chemicals, but when herbivorous insects damage a plant, many more volatiles are released and the chemical identity of the volatile compounds varies with the plant species and with the herbivorous insect species. These volatiles attracted both parasitic and predatory insects that are natural enemies of the herbivores. In addition to the bouquet of compounds that render leaves attractive or disagreeable to herbivores, volatile terpenoids and other compounds emitted from leaves in response to insect damage in *P. fluorescens* treated plants allow insect parasitoids (such as parasitic wasps) and predators to distinguish between infested and noninfested plants and thus aid in locating hosts or prey. The corn and cotton plants release volatiles that attract hymenopterous parasitoids to attack larvae of several lepidopteran species (Tumlinson *et al.*, 1993). The structural uniformity in the chemical emissions from bacterized plants with insect feeding suggests the activation of a set of biosynthetic pathways shared by a wide range of plant families and that the products are detectable to a broad spectrum of insect parasitoids and predators. In the same way, *P. fluorescens* treatment in the rice plants might have enhanced and mimicked the herbivory.

The bacterized plant's ability to differentiate between herbivore damage and a general wound response suggest the presence of elicitors associated with insect feeding that are absent from other types of leaf damage. Although the volatile compounds released by insect herbivore damage are similar among the several plant species studied, the specific blends are quite distinct, varying in both the numbers of compounds, quantity and the actual structures produced. In tobacco, higher concentrations of the defense-signaling molecule jasmonic acid result from herbivore damage by hornworm caterpillars than from mechanical damage designed to mimic herbivory (McCloud and Baldwin, 1998).

In the present study, it was clear that the application of talc based formulation containing either single strain (PF1 or FP7) or mixture of both strains (with or without chitin) reduced the sheath blight incidence and leaffolder infestation both under

greenhouse and field condition through induced systemic resistance. The onset of ISR against pest and disease associated with increased accumulation of defense enzymes, viz., PAL, chitinase PO, PPO, increased amount of phenol and accumulation of lignin. In addition, *P. fluorescens* bacterization promoted the plant growth and attracted the natural enemies of leaffolder and under field condition.

The field consistency of the strains used in this study were already verified in different locations against sheath blight. However our study is the first report in India that, *P. fluorescens* can be used for both leaffolder and sheath blight management. However, these strains have to be further tested in different ecological situations against leaffolder to confirm the consistent performance of the strains.

In conclusion, *P. fluorescens* reduced the sheath blight development and leaffolder infestation and increased plant growth, which ultimately increased the grain yield in rice.

Summary

CHAPTER VI

SUMMARY

The present study was taken up to assess the *Pseudomonas* mediated induced systemic resistance against sheath blight and leaffolder in rice under glasshouse and field conditions.

Pseudomonas strains (PF1 and FP7) increased the germination of rice seeds, shoot and root length of seedling and ultimately vigour index. Both the strains PF1 and FP7 either with or without chitin amendment in the talc based formulation increased root and shoot length significantly in all the varieties tested.

Application of *P. fluorescens* strains either as individual or as strain mixture (with and without chitin) significantly suppressed the sheath blight and leaffolder incidence in glasshouse grown rice plants.

In leaffolder bioassay, *Pseudomonas* treated leaves greatly altered the feeding preference of the larvae though chemical treated plants were effective in affecting the feeding rate than the bacterized plant. Reduction in larval and pupal weight, increase in larval mortality and adult malformation were observed in bacterized leaves under *in vitro* test. The larval mortality was found to be similar in both PF1 and FP7 treated plants. The strain mixture resulted in increased mortality than the individual strains.

Increase in the activity of defense enzymes *viz.*, chitinase, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase and phenol and lignin content were found to be significant in *P. fluorescens* treated rice plants challenged with pathogen and insect as compared to control plants.

In *P. fluorescens* strain mixture treated plants, 2.0 to 2.5 fold increased chitinase activity over control was recorded in the leaf folder and sheath blight inoculated plants respectively.

Two chitinase isoforms with molecular weight of 28 and 35 kDa in rice sheath and 35 and 56 kDa in rice leaves were detected in *Pseudomonas* treated plants inoculated with *R. solani* and *C. medinalis* respectively. The two-chitinase isoforms of rice sheath and leaves were also found to be expressed constitutively in *Pseudomonas* treated plants and untreated plants. However the activity was more pronounced in plants challenge inoculated with pest and pathogen. The purified 35 kDa chitinase from plants treated with mixture of strains treatment (PF1+FP7) inhibited the growth of *R. solani* under *in vitro*.

Four peroxidase isozymes (PO1-PO4) and five isozymes (PO1-PO5) were detected in strain mixture treated plant, which was challenge inoculated with *R. solani* and *C. medinalis* respectively.

The activity of PAL in the sheath and leaves of bacterized rice plants inoculated with pathogen and insect was at least 2 fold higher than the control plants.

A unique *Pseudomonas* induced PPO2 isoform was detected in gels of extracts prepared from leaf tissues as compared to control plants.

Treatment of rice plants with strain mixture induced more accumulation of phenol. Increased lignin content was recorded in the *Pseudomonas* treated plants particularly in the mixture treatment at seven days after inoculation of the sheath blight pathogen and leaf folder.

The bacterial growth of the strains varied significantly in the media amended with chitin from the media without chitin. The bacterial population was higher in the PF1+FP7 inoculated medium amended with chitin. The FP7 strain showed increased chitinase activity than the PF1 strain.

The application of talc-based formulation containing either individual or mixture of *Pseudomonas* strains significantly reduced the sheath blight and leaf folder incidence under two field experiments. Maximum of 62.4% and 62.0% disease reduction was noticed in mixture treatment in trial I and II respectively. However, in the case of leaf folder, maximum control (80.0 % and 73.3%) was achieved by application of chlorpyrifos in trial I and II followed by *Pseudomonas* strain mixture (47.7% and 56.1%).

Increased plant height and tiller number/hill were observed in the *P. fluorescens* treated plants, which eventually increased the grain yield. The highest grain yield of 6.53 and 5.04 tonnes/ha was observed in plots bacterized with strain mixture + chitin as against the control plot which recorded only 5.8 and 3.84 tonnes/ha in trials I and II respectively. The yield was also significantly greater than the untreated control when bacterized with individual strains PF1 (6.28 and 4.64) and FP7 (6.21 and 4.55).

The occurrence of natural enemy (hymenopterous parasitoid and spider) population increased invariably in *Pseudomonas* treated plants as compared to chemical and control treatments.

In all the experiments of this study, strain mixture performed better than their respective single strain and also the chitin amendment in the talc- based formulation containing either single strain or strain mixture considerably increased the biocontrol activity of *Pseudomonas* against sheath blight and leaf folder of rice.

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Annexure

ANNEXURE

Media

Potato Dextrose Agar (PDA) (Riker and Riker, 1933)

Peeled potato	:250.0 g
Dextrose	:20.0 g
Agar	:15.0 g
Distilled water	:1000ml
pH	6.5

King's B medium (Kings *et al.*, 1954)

Peptone	: 20.0 g
Dipotassium hydrogen phosphate	: 1.5 g
Magnesium sulphate	: 1.5 g
Glycerol	: 10.0 ml
Agar	: 15 g
Distilled water	: 1000 ml

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 (1 g) 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 vacuum 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.*, 1955).

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.

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis
(SDS-PAGE) (Laemmli, 1970)**

Acrylamide stock

Acrylamide	: 30.00 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 μ l
10% APS (Ammonium per sulphate)	: 38 μ l
TEMED (N, N, N', N'-tetra methyl ethylene diamine)	: 5 μ l

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)	: 50 μ l
10% APS (Ammonium per sulphate)	: 25 μ l
TEMED (N, N, N', N'-tetra methyl ethylene diamine)	: 5 μ l

Sample buffer

Glycerol	: 20% (v/v)
Tris buffer, pH 6.8	: 0.125 M
Na ₂ EDTA	: 5 mM
SDS	: 20% (w/v)
Bromophenol blue	: 0.1% (w/v)
2-mercaptoethanol	: 1% (v/v)

Electrode buffer

Glycine	: 4.320 g
Tris base	: 0.900 g
SDS	: 0.300 g

(Contents dissolved in 200 ml distilled water and the volume were made up to 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

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% APS (Ammonium per sulphate)	: 25 µl
TEMED (N, N, N', N'-tetra methyl ethylene diamine)	: 5 µl

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% APS (Ammonium per sulphate)	: 25 μ l
TEMED (N, N, N', N'-tetra methyl ethylene diamine)	: 5 μ l

WESTERN BLOTTING (Gallagher *et al.*, 1995)

Anode buffer I

Tris base	: 18.17 g
Methanol	: 100 ml
Distilled water	: 350 ml
pH	: 10.4

Anode buffer II

Tris base	: 1.51 g
Methanol	: 100 ml
Distilled water	: 500 ml
pH	: 10.4

Cathode buffer

Tris base	: 1.51 g
6-amino hexanic acid	: 2.62 g
Methanol	: 100 ml
Distilled water	: 500 ml
pH	: 9.4

Tris buffered saline (TBS): 10x stock

100 mM Tris base	: 6.05 g
1.4 M NaCl	: 40.91 g
Distilled water	: 500 ml
pH	: 7.9

(Contents dissolved in 200 ml distilled water, pH adjusted with NaOH/HCl and the volume was made up to 500 ml with distilled water in all the buffers and stock).

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

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

