# PROTEOME ANALYSIS OF INDUCED SYSTEMIC RESISTANCE MEDIATED BY PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) IN RICE FOR BIOTIC STRESS

By

**Shinde Subhashini Ganesh**
**(2011-11-103)**

## THESIS

**Submitted in partial fulfillment of the**

**requirement for the degree of**

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**COLLEGE OF HORTICULTURE**
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# DECLARATION

I, hereby declare that this thesis entitled **"Proteome analysis of induced systemic resistance mediated by plant growth promoting rhizobacteria (PGPR) in rice for biotic stress"** is a bonafide record of research work done by me during the course of research and that it has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

Vellanikkara
Date: 21/08/2013

**Shinde Subhashini Ganesh**
(2011-11-103)

## CERTIFICATE

We, the undersigned members of the Advisory Committee of **Miss. Shinde Subhashini Ganesh** a candidate for the degree of **Master of Science in Agriculture**, with major field in **Plant Biotechnology**, agree that the thesis entitled **"Proteome analysis of induced systemic resistance mediated by plant growth promoting rhizobacteria (PGPR) in rice for biotic stress"** may be submitted by Miss. Shinde Subhashini Ganesh, in partial fulfillment of the requirement for the degree.

31/08/2013

**Dr. Abida P. S.**
(Chairman, Advisory Committe)
Associate Professor
Centre for Plant Biotechnology and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur

31/08/2013

**Dr. P. A. Valsala**
(Member, Advisory committee)
Professor and Head
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur

31/8/2013

**Dr. P. A. Nazeem**
(Member, Advisory committee)
Professor and Coordinator (DIC)
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur

31/8/13

**Dr. Sally. K. Mathew**
(Member, Advisory Committee)
Professor
Department of Plant Pathology
College of Horticulture
Vellanikkara, Thrissur

31/8/13

**Dr. A. Haseena Bhaskar**
(Member, Advisory Committee)
Associate Professor
Department of Agricultural
Entomology
Vellanikkara, Thrissur

31/8/13

**Dr. Deepu Mathew**
(Member, Advisory Committee)
Associate Professor
Centre for Plant Biotechnology and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur

# CERTIFICATE

Certified that this thesis entitled **"Proteome analysis of induced systemic resistance mediated by plant growth promoting rhizobacteria (PGPR) in rice for biotic stress"** is a record of research work done independently by **Miss. Shinde Subhashini Ganesh** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma or fellowship to her.

31/08/2013

Vellanikkara
Date: 31/08/2013

**Dr. Abida P. S.**
(Major advisor, Advisory committee)
Associate Professor
Centre for Plant Biotechnology and
Molecular Biology
College of Horticulture
Vellanikkara, Thrissur

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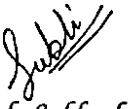

*Shinde Subhashini Ganesh*


To my
Aai-Baba

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## ABBREVIATIONS

| | |
|---|---|
| ABA | Abscisic acid |
| β | Beta |
| BTH | Benzothiadiazole |
| $^0C$ | Degree Celsius |
| cm | Centimeter |
| CPBMB | Centre for Plant Biotechnology and Molecular Biology |
| ET | Ethylene |
| g | Gram |
| h | Hours |
| HAI | Hours after inoculation |
| $H_2O_2$ | Hydrogen peroxide |
| ISR | Induced systemic resistance |
| JA | Jasmonate |
| KAU | Kerala Agricultural University |
| kDa | Kilo Dalton |
| M | Mole |
| mg | Miligram |
| min | Minute |
| ml | Millilitre |

| | |
|---|---|
| µg | Microgram |
| µl | Microlitre |
| OD | Optical Density |
| PAGE | Polyacrylamide Gel Electrophoresis |
| *Pf* | *Pseudomonas fluorenscens* |
| pH | Hydrogen ion concentration |
| % | Percentage |
| rpm | Rotations per minute |
| SA | Salicylic acid |
| SDS | Sodium dodecyl sulphate |
| sec | Second (s) |
| V | Volts |
| v/v | Volume by Volume |
| w/v | Weight by Volume |

# *Introduction*

# 1. INTRODUCTION

Rice (*Oryza sativa* L.), a native to South-East Asia is one of the leading food crops of the world and is second only to wheat in terms of annual production for food use. It is the main staple food for about 60 per cent of the world's population. Rice is predominantly an Asian crop, 95 per cent of it is being produced and consumed in the Southeast Asian countries extending from Indo-Pakistan sub-continent to Japan.

Rice is the most important food crop of India. Nearly three-fourth of the people in the country subsist on it. India has the largest area under rice in the world, but ranks second in production after China. It is grown in India under diverse agro-climatic conditions including irrigated, upland and lowland conditions. In India, rice is grown in an area of 449.7 lakh hectares, with a production of 905.5 lakh tonnes (Mathur *et al*, 1999). Occurrence of biotic and abiotic stresses has emerged as the major cause of rice yield instabilities across diverse crop growing areas. Disease and pest causes 35-40 per cent annual yield loss in rice (Shrinivasacharya, *et al*., 2002) and among the various diseases and pests in rice, sheath blight disease and brown planthopper insect pest causes severe yield losses globally.

Rice is an important cereal crop affected by various fungal, bacterial and viral diseases. Sheath blight caused by *Rhizoctonia solani* is emerging as a very destructive disease under favorable weather conditions in rice growing areas of the world, which ultimately causes substantial yield losses (Gautam *et al*, 2003). Management of this disease is difficult due to viability of sclerotia in the soil for several years. Use of fungicides to control the disease causes several adverse effects such as development of resistance in the pathogen, residual toxicity, pollution in the environment, high cost. Therefore, it has become necessary to adopt ecofriendly approaches for better crop health and for yield.

Among the pest, brown planthopper (*Nilaparvata lugens*) is one of the most destructive pests of rice throughout Asia. It causes severe yield reduction by directly sucking the plant sap and acting as a vector of viral diseases such as rice grassy stunt and

ragged stunt. Brown planthopper (BPH) is re-emerging as a key pest threatening rice production in tropical Asia. The salt stress in association with the incidence of BPH is becoming the major threat for rice production in coastal/inland saline areas in India. The adults are light or dark brown in colour with short winged and long-winged forms in both the sexes. They are found in clusters at the base of the plants. Both nymphs and adults suck sap from the plant tissues. Excess draining of the plant sap results in wilting and drying of the plants in clusters known as "hopper burn". It starts in circular patches and then covers the whole field.

Control of wide spectrum of pathogens and pests through biological mean is accepted as durable and environment friendly alternative for chemical based pest management. Plant growth-promoting rhizobacteria (PGPR) suppress a variety of root and vascular diseases caused by soilborne pathogens (Defago and Haas, 1990). The PGPR suppress disease by antagonizing pathogens via different mechanisms such as antibiosis, competition for iron or carbon, and production of lytic enzymes. PGPR also activate plant defense resulting in systemic protection against different fungal, bacterial, and viral pathogens (Alstrom, 1991), a phenomenon termed induced systemic resistance (ISR) (Kloepper *et al.*, 1992). *Pseudomonas fluorenscens* are Gram-negative rod shaped bacteria that inhibt soil, plants and water surfaces. The optimum growth temperature is between 25-30$^0$C Soluble, green fluorescent pigments are produced by *Pseudomonas fluorenscens* when the iron concentration is low. The significance of these organisms has increased because of their ability to degrade various pollutants and their use as biocontrol agent against pathogens.

In 1991, an alternative approach to inducing systemic resistance was reported by Alstrom (1991), Van Peer *et al.* (1991), and Wei *et al.* (1991). These authors independently demonstrated that selected strains of nonpathogenic plant growth-promoting rhizobacteria, which colonize the rhizosphere of the plant, are able to elevate plant resistance. Until then, these bacteria, mainly fluorescent *Pseudomonas* spp., had been studied for their ability to control soilborne pathogens through competition for nutrients, siderophore-mediated competition for iron, or antibiosis (Bakker *et al.,* 1991; Schippers 1992; Thomashaw and Weller 1995).

Induced Systemic Resistance (ISR) is a phenomenon where by resistance to infectious disease is systemically induced by localized infection or treatment with microbial components or products or by a diverse group of structurally unrelated organic and inorganic com-pounds. The activity of the inducing agents is not due to antimicrobial activity per se or their ability to be transformed into antimicrobial agents. However, antimicrobial agents can induce resistance, and they provide protection from the time of application until ISR is fully expressed. The process by which the plant becomes resistant is active. Events are put in motion and compounds are synthesized and accumulated which may contribute to resistance and the plant is sensitized to further respond rapidly after infection (Sticher *et al.*, 1997). ISR operates through the activation of multiple defense compounds at sites distant from the point of pathogen and pest attack. These induced responses are regulated by a network of interconnecting signal transduction pathways (salisalic acid (SA), jasmonic acid (JA) and ethelyne (ET) which play key role in activating defense.

Induction of systemic resistance in the plant thus appeared to be an additional mechanism by which these bacteria could protect the plant against disease. Induced systemic resistance (ISR) (Kloepper *et al.*, 2004) mediated by nonpathogenic rhizobacteria has been demonstrated in several plant species (Pieterse *et al.*, 1996 b) and shown to be effective against bacterial, viral, and fungal diseases. Maurhofer *et al.*, (1994) showed that ISR induced by *P.fluorescens* strain CHA0 in tobacco is associated with PR protein accumulation, suggesting that nonpathogen-induced ISR and pathogen-induced SAR share similar mechanisms.

With this background, the present study was undertaken to analyze proteome with special reference to biotic stress tolerance in a popular rice variety of Kerala, Jyothi which is susceptible to sheath blight disease and brown planthopper pest, through application of Pseudomonas fluorenscens.

# Review of Literature

# 2. REVIEW OF LITERATURE

## 2.1. Introduction

Rice (*Oryza sativa* L.), a native to South-East Asia is one of the leading food crops of the world and is second only to wheat in terms of annual production for food use. It is the main staple food for about 60 per cent of the world's population. Rice is predominantly an Asian crop, 95 per cent of it is being produced and consumed in the Southeast Asian countries extending from Indo-Pakistan sub-continent to Japan.

Rice (*Oryza sativa* L.) is the most important staple food crop for more than two third of the population of India and more than 65 per cent of the world's population (Mathur *et al.*, 1999). Rice (*Oryza sative* L.) is the most important cereal crop in the tropics and is the staple food for over half of the world's population by providing 23 per cent of total calories consumed worldwide. Asian countries *viz.*, China, India, Japan and Korea produce and consume 90 per cent of the global rice and accounts for 60 per cent of daily caloric intake and rice production is severely affected due to pest and disease incidence. High-troughput methods for studying differential gene transcription or proteomics approaches for identifying the protein in relation to induced resistance in rice plants against pest and diseases are available. The literature pertaining to the present investigation has been reviewed under following heading : Importance of BPH and sheath blight pathogen in rice, PGPR and ISR against major pest and diseases.

## 2.2. Constraints to rice cultivation

The productivity of rice is threatened by pathogens and insects pests attacking the crop from nursery to harvest causing enormous yield loss. Among the pathogens, *R. solani* causing sheath blight disease receives major importance because of the occurrence in all the stages of the crop, thereby causing severe yield loss (Ou, 1985). The brown planthopper (*Nilaparvata lugens*) gained major importance because of its ability to cause considerable physiological damage to the rice plants by removing nutrients and

disrupting physiological processes and consequently the growth and development of the plant was affected (Watanabe and Kitagawa, 2000).

### 2.3. Importance of sheath blight

*Rhizoctonia solani* Kuhn. is the causal agent of rice sheath blight, which has become a major constraint to rice production during the last two decades . The emergence of *R. solani* as an economically important rice pathogen has been attributed to the intensification of rice-cropping systems with the development of new short-statured, high-tillering, high yielding varieties, high plant densities, and an increase in nitrogen fertilization (Ou, 1985).

Sheath blight, caused by *R. solani* occurs throughout the rice producing areas of the world and is second only to rice blast as most economically important fungal disease of rice (Ou, 1985). The natural infection of sheath blight disease occurs at the seedling, tillering and booting stage of rice. Infection usually starts near the water line of rice plant in paddy fields. Lesion develops upward to the upper leaf sheath and leaf blades .The centre of lesions becomes grayish white with brown margin, later several spots coalesce and show blight symptoms (Ou, 1985). Thus entire plant often gets killed under severe cases (Rush and Lindberg, 1984). The symptom of this disease suggests the possible involvement of phytotoxin in lesion development (Vidhyasekaran *et al,* 1997a; Sriram *et al.*, 1997). Attempt to control sheath blight with resistant cultivars have not been successful because of low levels of available host resistance.

Sheath blight disease was not given much attention before the advent of semi-dwarf varieties in the 1980's. The modern semi dwarf varieties are normally grown at high densities that create a favorable environment for development of sheath blight, one of the most important constraints to high grain yield and quality.

### 2.4. Importance of brown planthopper

The brown planthopper (BPH), *Nilaparvata lugens* is dimorphic, and causes direct damage to the rice crops and susceptible rice varieties often suffer severe yield losses annually from BPH infections (Sogawa *et al.*, 2003). This pest causes draining

out of plant fluid and nutrients by continuous sucking during high infestation and plants become yellow and finally die. This symptom of crop damage is known as hopper burn. They also transmit two viruses rice ragged stunt virus (RRSV) and rice grassy stunt virus (RGSV). Feeding behavior of this insect is highly specific to phloem sucking, as the phloem contain sucrose, potassium and amino acids as its main constituents (Hayashi and Chino, 1990). Such a feeding activity causes physiological damage to the rice plant by removing nutrients and disrupting physiological processes, and consequently affects the growth and development of the plant (Watanabe and Kitagawa, 2000). Moreover feeding by large number of BPH results in yellowing, drying of leaves, stunting and wilting of the tillers, which is referred to as hopper-burn condition (Michell and Maddison,1983) BPH also causes indirect damage by transmitting viruses such as the rice tungro, ragged stunt and grassy stunt viruses, which results in development of severe disease (Heinrichs, 1979). At this level, the loss is considered to be 100 per cent (Pathak, 1994)

The BPH is a good example of pest resurgence and the intensive use of insecticide caused the outbreak of the brown planthopper. Kenmore *et al.* (1984) reported that the population of the brown planthopper in the insecticide treated field was much higher than in the untreated field. The application of broad spectrum insecticides can selectively destroy natural predators and increase BPH densities a thousand-fold compared with no pesticide application.

## 2.5. Importance of PGPR on pest/disease management

Biological control by antagonistic organisms has been studied extensively and rhizobacterial strains have emerged as potential biocontrol agents for the control of root and foliar disease (Raupach and Kloepper, 1998; Ramamoorthy *et al.*, 2002b). The PGPR are also having the ability to protect above ground plant parts against viral, fungal and bacterial diseases by induced systemic resistance (ISR) (Kloepper *et al.*, 1992). Among the PGPR, fluorescent pseudomonads are the most exploited bacteria for biological control of soilborne and foliar plant pathogens.

The species of fluorescent pseudomonads are grouped into different biovars and subgroups based on similarity in biochemical tests (Champion *et al.*, 1980)

### 2.6. 1. Interaction between plants and rhizobacteria

The term "rhizosphere" is defined as a volume of soil surrounding plant roots in which bacterial growth is stimulated (Sorensen, 1997). The rhizosphere has attracted much interest since it is a habitat in which several biologically important processes and interactions take place. The rhizosphere is populated by a diverse range of microorganisms, and the bacteria colonizing this habitat are called rhizobacteria (Schroth and Hancock, 1982). Root exudates are believed to determine which microorganisms colonize roots in the rhizosphere (Kunc and Macura, 1988). It is now known that plant roots also generate electrical signals; it has been shown that zoospores of oomycetic pathogens take advantage of these signals to guide their movements towards the root surface (Gow *et al.*, 1999). It has been estimated that about 30 per cent of plant photosynthate production is released via root exudation (Smith *et al.*, 1993). Both passive leakage and active secretion are involved (Rougier and Chaboud, 1989). Leakage involves low molecular weight compounds release while secretion usually involves high molecular weight compounds that are actively transported across the cell membranes (Rougier and Chaboud, 1989). Composition and extent of exudation are determined genetically (Bolton *et al.*, 1993). This involves a certain cost for the plant, and therefore must provide a selective advantage. It has often been suggested that root exudation evolved in plants as a means to stimulate active micro flora (Bolton *et al.*, 1993). Exudation can provide both physical and chemical benefits to plants, such as root mucilages reduce friction between root tips and the soil and reduce root desication, improve the contact between the root and the soil, and contribute to soil structural stability (Rougier and Chaboud, 1989).

### 2.6.2. Plant root colonizing bacteria

Root exudates also attract microorganisms (Jaeger *et al.*, 1999; Lemanceau *et al.*, 1995). Conversely, rhizobacteria can also elicit root exudation (Bolton *et al.*, 1993) in a species-specific manner such as metabolites produced by *Pseudomonas aeruginosa* stimulated root exudates by perennial ryegrass twelve-fold (Merharg and Killham, 1995).

### 2.6.3. Plant growth promoting rhizobacteria

Plant root colonizing bacteria can function as harmful, deleterious rhizobacteria (DRB) or beneficial, plant growth promoting rhizobacteria (PGPR). Rhizobacteria that inhibit plant growth have been described as deleterious rhizobacteria (Suslow and Schroth, 1982). Plant growth promotion by rhizobacteria can occur directly and indirectly (Glick, 1995; Persello-Cartieaux *et al.*, 2003). There are several ways by which plant growth promoting bacteria can affect plant growth directly, by fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus, production of siderophores that solubilize and sequester iron, or production of plant growth regulators (hormones) that enhance plant growth at various stages of development. Indirect growth promotion occurs when PGPR promote plant growth by improving growth restricting conditions (Glick *et al.*, 1999). This can happen directly by producing antagonistic substances, or indirectly by inducing resistance to pathogens (Glick, 1995). A bacterium can affect plant growth by one or more of these mechanisms, and also use different abilities for growth promotion at various times during the life cycle of the plant (Glick *et al.*, 1999).

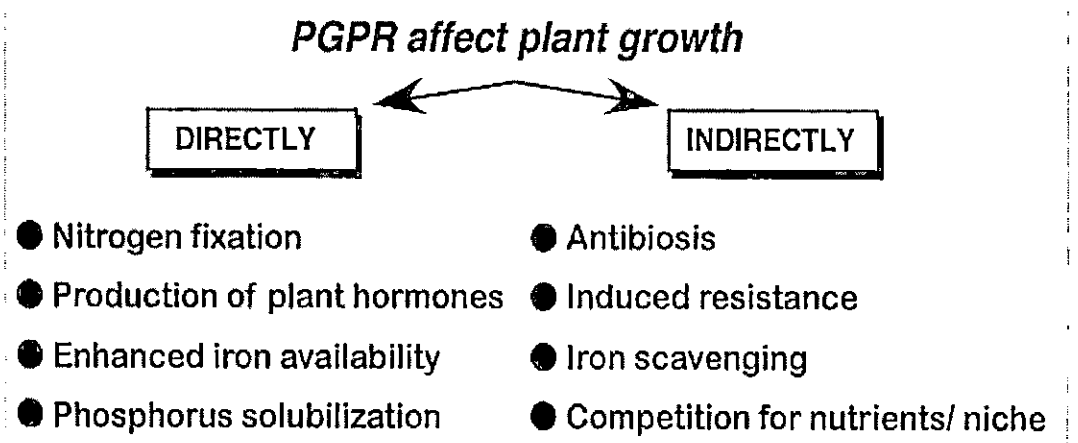



**Fig.1. Plant growth promoting rhizobacteria affecting plant growth directly and indirectly**

### 2.6.3.1. Direct plant growth promotion

The ways by which PGPR can influence plant growth directly may differ from species to species as well as from strain to strain. Symbiotic plant colonizers such as rhizobia mostly contribute to plant growth by nitrogen fixation. Free-living rhizobacteria usually do not rely on single mechanisms of promoting plant growth (Glick *et al.*, 1999). In addition to nitrogen fixation, several PGPR are also able to provide the plant with sufficient iron in iron-limited soils (Wang *et al.*, 1993), or other important minerals, like Phosphate (Singh and Singh, 1993). Organic substances capable of regulating plant growth produced either endogenously or applied exogenously are called plant growth regulators. They regulate growth by affecting physiological and morphological processes at very low concentrations (Arshad and Frankenberger, 1998). Several microorganisms are capable of producing auxins, cytokinins, gibberellins, ethylene (ET), or abscisic acid (ABA). Auxins are produced by several rhizobacterial genera, such as *Azospirillum, Agrobacterium, Pseudomonas,* and *Erwinia* (Costacurta and Vanderleyden, 1995). In the case of *Azospirillium*, bacterial colonization takes place in the zone of lateral root emergence. *Azospirillum* inoculation increases the density and length of root hairs as well as the elongation rates of lateral roots, increasing the root surface area (Dobbelaere *et al.*, 2001; Fallik *et al.*, 1994)

Ethylene, a hormone produced in all plants, mediates several responses to developmental and environmental signals in plants. Its involvement in plant growth when excreted around the roots has also been shown (Arshad and Frankenberger, 1998). The PGPR *Pseudomonas putida* GR12-2 stimulates plant root elongation (Glick *et al.*, 1994). Mutant strains lacking aminocyclopropane carboxylic acid (ACC) deaminase activity were unable to promote root elongation of canola seedlings; this enzyme hydrolyzes ACC, the immediate precursor of ET in plants Cytokinins and gibberellins are produced in the rhizosphere by several bacteria, such as *Azospirillum, Agrobacterium,* and *Pseudomonas* genera (Gaudin *et al.*, 1994). Cytokinins promote root formation, but a minor overproduction of cytokinins instead leads to inhibition of root development, and severely deficient cytokinin mutant plants do not survive (Binns, 1994).

### 2.6.3.2. Indirect plant growth promotion

Several PGPRs are known to reduce the effects of plant stresses by limiting phytopathogen caused damage. This can occur by local antagonism of soilborne pathogens, or by induction of systemic resistance against pathogens throughout the entire plant.

#### 2.6.3.2.1. Biocontrol of soilborne pathogens

Over the last decades, a great diversity of rhizospheric microorganisms has been described, characterized, and in many case tested for activity as biocontrol agents against soilborn pathogens. Such microorganisms can produce substances that may limit the damage caused by phytopathogens, by producing antibiotics, siderophores, and a variety of enzymes. These microorganisms can also function as competitors of pathogens for colonization sites and nutrients. Nevertheless, biocontrol has not yet become widely applied, for several reasons. The efficiency of a biocontrol strain under field conditions is likely to be affected by several environmental conditions such as pH, temperature, water content, and interactions with other microorganisms. Also, some biocontrol agents that showed promising traits in initial experiments failed to be efficient rhizosphere colonizers under more complex biological conditions. This argues that it is worthwhile to address these limitations, and the genetic, biochemical, and physiological factors that contribute to the activity of biocontrol agents, by careful studies (Dobbelaere *et al.*, 2001).

#### 2.6.3.2.2. Antibiotic production

In many biocontrol systems, one or more antibiotics have been shown to play a role in disease suppression. Molecular tools have been effective here, because mutants defective in antibiotic production are easily obtained, and *in vitro* assays are useful tests. The most widely studied group of rhizospheric bacteria with respect to the production of antibiotics is that of the fluorescent Pseudomonads. The first antibiotics described as being implicated in biocontrol were phenazine derivatives produced by fluorescent pseudomonad (Weller and Cook, 1983). Their role has been elucidated by transposon insertion mutations which result in a defect in production of phenazine-1-carboxylate, thus reducing disease suppressive activity (Pierson and Pierson, 1996). The genes

encoding the enzymes responsible for synthesis of the metabolites have been isolated and their regulation studied (Bangera and Thomashow, 1996; Pierson *et al.*, 1995). Global regulatory elements have been shown to coordinate the production of these metabolites (Pierson *et al.*, 1994). The presence of populations of other bacteria can influence phenazine production by *P. aureofaciens,* since mutants lacking the ability to produce an auto inducer signal required for induction of antibiotics synthesis can use auto inducers produced by other (related) rhizosphere inhabitants (Pierson and Pierson, 1996; Wood and Pierson, 1996).

**2.6.3.2.3. Siderophores**

Iron is abundant in the Earth's crust but most of it is in the highly insoluble form of ferric hydroxide and thus unavailable to organisms in soil solution. Some bacteria have developed iron uptake systems (Neilands and Nakamura, 1991). These systems involve a siderophore – an iron binding ligand and an uptake protein, needed to transport iron into the cell. It has been suggested that the ability to produce specific siderophores, and to utilize a broad spectrum of siderophores, may contribute to the root colonizing ability of *Pseudomonas* strains. The production of siderophores that chelate and thereby scavenge, the ferric iron in the rhizosphere, may result in growth inhibition of other microorganism whose affinity for iron is lower (Kloepper *et al.*, 1988).

Siderophore mechanisms will only be relevant under conditions of low iron availability. As soil pH decreases below 6, iron availabity increases and siderophores become less effective (Neilands and Nakamura, 1991). Optimal suppression of pathogens occurred at levels between $10^{-19}$-$10^{-24}$ M. The critical level of iron at which a siderophore-producing strain of *Pseudomonas putida* suppressed the growth of a fungal pathogen, Fusalicyclic acidrium oxysporium, was found to be < $10^{-16}$ M (Neilands and Nakamura, 1991). As the synthesis of each siderophore generally requires the activity of several gene products (Mercado-Blanco *et al.*, 2001), it is difficult to genetically engineer bacteria to produce modified siderophores. Complementation studies of siderophore-deficient mutants of *P. fluorescens* M114 indicated that at least five separate genetic loci

are needed to encode the enzymes involved in the synthesis of the siderophore pseudobactin M114 (O'Sullivan *et al.*, 1990).

**2.6.3.2.4. Parasitism**

An additional mechanism by which biocontrol agents can reduce plant diseases is biocontrol agent parasitism on pathogen s, mostly fungi. Digestion of the parasite cell wall is accomplished by several excreted enzymes including proteases, chitinases and glucanases. Individually, all these enzymes display antifungal activity, but they often act synergistically with antibiotics (Lorito *et al.*, 1993; Lorito *et al.*, 1994).

**2.6.3.2.5. Competition for nutrients and niches**

In addition to the above described and commonly reported antibiosis mechanisms there are other ways by which rhizobacteria can inhibit pathogens. One example concerns competition for nutrients and suitable colonization niches on the root surface. Such mechanisms are often overlooked, in part because they are difficult to study in biological systems. Competition for nutrients supplied by root exudates is probably a significant factor in most interactions between PGPR and pathogens. Populations of bacteria established on a plant root could act as a sink for the nutrients in the rhizosphere, hence reducing the nutritional element availability for pathogen stimulation or subsequent colonization of the root. This mechanism is most probably often used by fluorescent pseudomonads due to their nutritional versalicyclic acidtility and because of their high growth rates in the rhizosphere (Sorensen, 1997).

**2.7. Biochemical and molecular characterization of fluorescent pseudomonads**

Based on the phenotypic and biochemical methods, the genus *Pseudomonads* is characterized as Gram-negative, rod shaped, aerobic and usually it is associated with plants. Krieg and Holt, (1984) identified some of the PGPR isolates based on physiological and nutritional tests. Most of the plant associated *Pseudomonads* sp. Belong to *P. fluorencens* and *P. putida* complex. There was no clear distinction between *P. fluorencens* and *P. putida* (Sheath *et al.,* 1981). However these two species are identified based on trehalose utilization and gelatin liquefaction. In this, *P. fluorencens*

exhibit positive for both the tests whereas *P.putida* shows negative response (Hildebrand *et al.,* 1992).

**2.8. Plant Growth Promotion**

The PGPR has significant impact on plant growth and development in two different ways, indirectly by decreasing or preventing some of the deleterious effects of a phytopathogenic organisms and directly by promoting plant growth through facilitating the uptake of nutrients from the environment (Glick *et al.,* 1999). Plants growth benefits due to addition of PGPR includes increase in germination rate, root growth, yield including grain, leaf area, chlorophyll content, magnesium, nitrogen and protein content, hydraulic activity, tolerance to drought and salt stress, shoot and root weight and delayed leaf senescence (Lucy *et al.,*2004). The PGPR mediated plant growth enhancement was reported by many workers (Van Peer and Schippers, 1988; Glick *et al.,* 1999; Kloepper *et al.,* 1988).

**2.9. Biocontrol activity by inducing systemic resistance**

Systemic resistance refers to an increased level of resistance at sites within that plant distant to those at which induction had occurred. How bacteria trigger systemic resistance is still largely unknown. Several sequential events have however been envisaged. A bacterial component, most likely a metabolite, is perceived by the plant root/leaf through binding to a receptor. This recognition mediates the extracellular signal to an intracellular signal. Thereafter, the metabolite itself, or a signal generated by the plant cell, initiates a cascade of signal transduction. Eventually, the translocated signal is perceived by distant plant cells, triggering the activation of the defense arsenal of the challenged host plant.

Induced resistance is also defined as an enhancement of the plant's defensive capacity against a broad spectrum of pathogens that is acquired after appropriate stimulation. The classic way of eliciting induced resistance is by a predisposal infection with a pathogen that causes a hypersensitive reaction. The resulting elevated resistance response upon challenge inoculation of plant parts distant from the site of primary

infection is known as systemic acquired resistance (SAR) (Hammerschmidt and Kud 1995). The induction of resistance by rhizobacteria is referred as Induced systemic resistance (ISR) (Van Loon *et al.,* 1998).

A large number of defense enzymes have been associated with ISR that include phenylalanine ammonia lyase, chitinase, β-1, 3-glucanase, peroxidase, polyphenol oxidase, SOD, CAT, APX, and protein inhibitors (Van Loon, 1994). Chitinases and β-1, 3-glucanase show sunergic antifungal activity and are related to the systemic acquired resistance (SAR). This enzymes involved in the liberation of molecule that elicit the first step of induction of resistance, phytoalexins and phenolic compounds (Van Loon *et al.,* 1994). ISR by PGPR has been achieved in large number of crops including *Arabidopsis* (Pieterse *et al.,* 1996), cucumber (Wei *et al.,* 1991), tomato (Duijff *et al.,* 1999), potato (Doke *et al.,*1987), carnation (Van Peer *et al.,*1991), and mango (Vivekananthan *et al.,* 2004) against broad spectrum of pathogens.

The PAL catalyses the deamination of *L*- phenylalanine to trans – cinnamic acid, which is the first step in biosynthesis of large class of plant products. The PAL is the key enzyme in inducing synthesis of salicylic acid (SA), which induces systemic resistance in many plants. The ISR activity of PAL was reported by Li *et al.* (1993), Chen *et al.* (2000), Radjacommare *et al.* (2002) in many crops.

### 2.9.1. Signaling in plants

Signal transduction pathways are activated upon microbial elicitor challenge leading in turn to activation of different sets of effecter molecules. The application of molecular, genetic and biochemical techniques had led to the identification of key components of the signaling pathways that result in defense responses in *Arabidopsis*. Signalling molecules like salicylic acid (SA) (Metraux *et al.*, 1990), jasmonate (JA) (Penninckx *et al.*, 1996), and ethylene (ET) (Boller, 1991), when accumulating, coordinate the defense responses and, when applied exogenously, are even sufficient to induce resistance (Ryals, 1996). It has been shown that these signalling molecules activate specific sets of defense-related genes: SA induces genes encoding pathogenesis-related proteins (prs) (Uknes *et al.*, 1992). These proteins have antimicrobial activity

(Kombrink and Somssich, 1995). The ET is involved in the expression of the genes encoding *Hel* (a heveine-like protein; (Potter *et al.*, 1993)), *chib* (basic chitinase; (Samac and Shah, 1994)), and *Pdf1.2* (a plant defensin; (Penninckx *et al.*, 1996)). Also JA has been shown to activate the genes encoding these three proteins (Penninckx *et al.*, 1996), all of which also possess antifungal activity. In addition, JA also activates the gene encoding a vegetative storage protein, *Atvsp* (Berger *et al.*, 1995). This protein accumulates in vacuoles, but its putative roll in defense activity has not been established. It has been reported that, in *Arabidopsis*, two general defense pathways are induced, induced systemic resistance (ISR) and systemic acquired resistance (SAR). ISR is a rhizobacterially mediated systemic resistance that does not involve any damage to plant. By contrast, SAR is induced by foliar pathogens and results in activation of resistance mechanisms in uninfected parts. Thus, in SAR a first infection predisposes the plant to resist further attacks. SAR induction is dependent on the accumulation of SA and requires the regulatory (activator) protein NPR1. Beside SA accumulation, several JA- and ET-dependent resistance mechanisms that are independent of SA have also been reported (Thomma *et al.*, 1998; Thomma *et al.*, 2001a). JA and ET act synergistically in inducing genes for several PR proteins (Norman-Setterblad *et al.*, 2000). The ET has been shown to *enhance* JA-dependent responses (Xu *et al.*, 1994), whereas SA *inhibits* the expression of JA– dependent defense genes (Gupta *et al.*, 2000; Vidal *et al.*, 1997). Similarly, JA has also been shown to interfere with SA-dependent signalling (Niki *et al.*, 1998). ISR can be triggered in plants which are unable to accumulate SA (*nahg* mutant plants). Based on this, one can conclude that SA is not required for ISR activation in *Arabidopsis*. Moreover, PR proteins do not usually accumulate in induced plants. However, the regulator NPR1 protein is required for expression of ISR (Pieterse *et al.*, 1996). *Arabidopsis* mutant plants in which the ET or JA-responsive genes *etr1, ein2, ein7, or jar1* are defect, thus conferring a reduced sensitivity to ET and JA, are also affected in their expression of ISR. Application of either ACC or JA to wild-type plants induces a resistance that is not associated with the accumulation of prs, but is dependent on a functional *npr1* gene. Treatment of the *jar1* mutant plant with ACC was effective in inducing resistance (Pieterse *et al.*, 1998). Application of JA to the *etr1*mutant, in contrast, did not elicit ISR (Pieterse *et al.*, 1998). Both compounds, ACC and JA, were

ineffective in inducing resistance in the *npr1* mutant, thus supporting the requirement for this key regulator in the response. These results indicate that responsiveness to JA and ET are required sequentially, and before NPR1, in the ISR signal transduction pathway (Pieterse *et al.*, 1998).

**2.9.1.1. Microbial signals involved in inducing resistance**

Both types of induced resistance are initiated by microbial signals which are perceived by the plant. Microbial signals in ISR rhizobacterially mediated systemically induced resistance is the result of a process that does not involve any obvious damage to the plant. For induction of ISR in radish, 10 colony forming units of the inducing *Pseudomonas sp.* Strain per gram 5 of root tissue was reported as a minimum threshold (Raaijmakers *et al.*, 1995). This level probably exceeds that found for individual bacterial strains in natural soils but can easily be obtained in more artificial systems.

The inducing bacteria are mostly saprophytic and can simultaneously induce resistance and promote plant growth (Pieterse and van Loon, 1999). In some cases, transposon mutagenesis experiments have indicated factors involved in induced resistance. It has been shown for three *Pseudomonas* strains that an outer membrane lipopolysaccharide (LPS) with strain specific O-antigenic carbohydrate side-chains is the elicitor (Leeman *et al.*, 1995). The purified siderophore of *P. fluorescens*, pseudobactin 374, could induce resistance in radish (Leeman *et al.*, 1996). It has also been shown that treatment of raddish plant roots even with nanogram quantities of SA was sufficient to induce systemic resistance against *Fusarium oxysporum* (Leeman *et al.*, 1996). *Arabidopsis* roots are capable of recognizing the presence of bacterial flagella. A receptor for bacterial flagellin was characterized in *Arabidopsis* roots (Gomez-Gomez and Boller, 2000); binding of flagellin leads to a reduction in root elongation (Gomez-Gomez *et al.*, 1999). As most resistance-inducing rhizobacteria promote growth and enhance root elongation (Glick *et al.*, 1999) the flagellin-induced growth inhibition must be compensated by other activities.

## 2.10. Fluorescent pseudomonads as PGPR

Fluorescent pseudomonads have emerged as the biggest and potentially the most promising group amongst the PGPRs involved in biocontrol of diseases (Suslow and Schroth, 1982).

Fluorescent pseudomonads are Gram negative, aerobic rods, motile with polar flagella and have the ability to produce water soluble yellow green pigment (Palleroni *et al.,* 1973). They comprise the species of *P. fluorescens* (four bio types), *P. putida* (two bio types), *P. aeruginosa, P. chlororaphis, P. aureofaciens* and *P. syringe* (Schippers *et al.,* 1987). They are well adapted to rhizosphere and rhizoplane, have a fast growth rate in the rhizoplane (Bowen and Rovira, 1976) and are able to utilize a large number of organic substrates (Stolp and Godkari, 1981) including root exudates.

The worldwide interest in this group of rhizobacteria was sparked off by the studies initiated at the University of California, Berkeley, USA during the 1970s. In 1978, Burr *et al.* (1978) reported that strains of *P. fluorescens* and *P. putida* applied to seed tubers improved the growth of potato. These findings were confirmed and later exemplified in the radish (Kloepper and Schroth, 1988), sugar beet (Suslow and Schroth, 1982).

Fluorescent pseudomonads are known to produce plant growth promoting substances like, auxins, gibberrelins, cytokinines etc (Suneesh, 2004). Thirty isolates of fluorescent pseudomonads from wheat rhizosphere were found to produce 1.1 to 12.1 mg of auxin per liter of medium without tryptophan and 1.8 to 24.8 mg per liter with tryptophan.

Suneesh (2004) recorded IAA and GA production in the range of 1.63 to 17.0 µg and 0.72 to 5.27 µg/ 25 ml broth by fluorescent pseudomonads isolated from moist deciduous forest of Western Ghats. The IAA and GA production by 52 fluorescent pseudomonads ranged from 80 to 760 µg and 24.82 to 262.8 µg of GA per liter of broth respectively.

Pal *et al.* (2003) reported four PGPRs strains belonging to fluorescent pseudomonads groups increased the root length, shoot length and pod yield of groundnut which were attributed to production of siderophores and IAA like substances. Two strains of fluorescent *Pseudomonas* sp. isolated from the potato epidermis and roots significantly increased growth of potato plants up to 500 per cent greater than control in green house assays

Tomato, cucumber, lettuce and potato plants bacterized with plant growth promoting *Pseudomonas* strain showed increased root and shoot fresh weight and simultaneous suppression of deleterious pathogenic microflora was observed (Vanpeer and Schippers, 1988).

Dileep Kumar and Dube (1992) reported that bacterization of chickpea and soybean seeds with a siderophore producing fluorescent pseudomonad RBT13 isolated from the tomato rhizoplane, resulted in increased seed germination, growth and yield of plants.

Seed treatment of radish with *Pseudomonas fluorescens* WCS 374 reduced the incidence of *Fusarium* wilt and increased the yield from 19.5 to 100 per cent in greenhouse conditions (Leeman *et al.*, 1995).

Plant growth promoting strains of *Pseudomonas fluorescens* ANP15 and *Pseudomonas aeruginosa* 7 NSK-2 were found to protect maize seeds from cold stock damage and significantly increased the seed germination as well as dry matter content of inoculated plants (Hofte *et al.*, 1991).

Pierson *et al.* (1994) tested the ability of fluorescent pseudomonas strains either alone or in combinations to suppress take all in fields infested with *Gaeumannomyces graminis* var *triticii*, and found the combined application of fluorescent pseudomonas strains to increase the yield by 20.4 per cent over untreated control and performed better over singly or individually applied treatments.

*Pseudomonas* species isolated from rhizosphere of fir and spruce plants in forest floor of Kashmir valley have been reported to increase the height, number of leaves, girth

and weight of fir and spruce plants significantly in addition to enhancing the nitrogen, phosphorous and potassium contents of plants (Zarger *et al.*, 2005).

Significant increase in seedling weight, mature root weight and total sucrose yield of sugar beet has been reported due to seed inoculation of fluorescent *Pseudomonas* sp. under field condition (Suslow and Schroth, 1982).

Vrany and Fiker (1984) recorded 4-30 per cent improvement in plant growth and tuber yield of potato inoculated with *P. fluorescens* under field conditions. Yuen and Schroth (1986) observed 18 - 41 per cent increase in the fresh top weight of carnation, sunflower, vinca and zinnia due to inoculation of *P. fluorescens* to the seeds or rooted cuttings.

Kaiser *et al.* (1989) studied effectiveness of seven strains of fluorescent pseudomonads to suppress seed rot and pre emergence damping off in chickpea caused by *Pythium ultimum* and found two strains of *Pseudomonas fluorescens, viz.*, Q29z – 80 and M8z-80, to increase emergence and yield of chickpea as compared to untreated controls.

Walley and Germida (1997) observed enhancement of shoot dry weight from 16 to 48 per cent and root dry weight from 82 to 137 per cent when inoculated with fluorescent pseudomonads.

Gupta *et al.* (2002) reported that peanut seeds bacterized with *Pseudomonas* GRC2 showed a significant increase in germination (83 per cent) under field conditions. Plant growth promoting fluorescent pseudomonad isolates, *viz.*, PGPR1, PGPR2 and PGPR4, significantly enhanced pod yield (23-26 per cent, 24-28 per cent and 18-24 per cent, respectively), haulm yield, nodule dry weight, root length, pod number, 100 kernel mass and shelling out turn over 3 years.

The yield of sunflower inoculated with *Pseudomonas* sp, *viz.*, PSI and PSII was increased by 32.8 per cent and 18.3 per cent, respectively over uninoculated control (Bhatia *et al.*, 2005).

## 2.11. Pathogenesis-related (PR) protein

The defense strategy of plants against stress factors involves a multitude of tools, including various types of stress proteins with putative protective functions. A group of plant-coded proteins induced by different stress stimuli, named "pathogenesis-related proteins" (PRs) is assigned an important role in plant defense against pathogenic constraints and in general adaptation to stressful environment.

The first PR- 1 protein was discovered in 1970. Since then, a number of PR-1 proteins have been identified in Arabidopsis, *Hordeum vulgare* (barley), *Nicotiana tabacum* (tobacco), *Oryza sativa* (rice), *Piper longum* (pepper), *Solanum lycopersicum* (tomato), *Triticum* sp. (wheat) and Zea mays (maize) (Liu and Xue, 2006). These PR-1 having 14 to 17 kD molecular weight and mostly of basic nature. Non-expressors of Pathogenesis-Related Genes1 (NPR1) regulate systemic acquired resistance via regulation of pathogenesis-related 1 (PR-1) in *Arabidopsis thaliana*. The interaction of nucleus-localized NPR1 with TGA transcription factors, after reduction of cysteine residues of NPR 1 by salicylic acid (SA) results in the activation of defense genes of PR-1. In the absence of TAG 2 and/or SA expression of PR-1 not occur in *Arabidopsis thaliana* (Despres *et al.*, 2000; Rochon *et al.*, 2006).

These PRs, defined as proteins coded for by the host plant but induced specifically in pathological or related situations (Van Loon *et al.,* 1994) do not only accumulate locally in the infected leaf, but are also induced systemically, associated with the development of systemic acquired resistance (SAR) against further infection by fungi, bacteria and viruses. Induction of prs has been found in many plant species belonging to various families (Van Loon, 1999), suggestive of a general role for these proteins in adaptation to biotic stress conditions. SAR, likewise, is a generally occurring phenomenon, that engenders an enhancement of the defensive capacity of plants in response to necrotizing infections (Sticher, *et al.*, 1997) There upon, in 1994 a unifying nomenclature for prs was proposed based on their grouping into families sharing amino acid sequences, serological relationship, and/or enzymatic or biological activity. By then

11 families (PR-1-11) were recognized and classified for tobacco and tomato (Van Loon *et al.*, 1994)

Pathogenesis-related proteins (PR-proteins) are plant species-specific proteins induced specifically in pathological or related situations. They are not only accumulated locally in the infected leaf, but are also induced systemically, and are associated with the development of systemic acquired resistance (SAR) against further infection by fungi, bacteria and viruses.( Sindelarova and Sindelar, 2005)

**2.11.1.α-Glucanase (PR2)**

Plant ß-1, 3-glucanases (ß-1,3-Gs) comprises of large and highly complex gene families involved in pathogen defense as well as a wide range of normal developmental processes. ß-1, 3-Gs have molecular mass in the range from 33 to 44 kDa (Hong and Meng, 2004; Saikia *et al.*, 2005).

These enzymes are found in wide variety of plants like *Arachis hypogaea* (peanut), *Cicer arietinum* (chickpea), *Nicotiana tabacum* (tobacco), etc. And having resistivity against various fungi like *Aspergillus parasiticus*, *A. Flavs, Blumeria graminis, Colletotrichum lagenarium, F. culmorum, F. oxysporum, F.udum, Macrophomina phaseolina* and *Treptomyces sioyaensis* (Rezzonico, 1998; Wu and Bradford, 2003; Hong and Meng, 2004; Wrobel-Kwiatkowska *et al.*, 2004, Liang *et al.*, 2005; Roy-Barman *et al.*, 2006). α -1,3-glucanases are involved in hydrolytic cleavage of the 1,3-α-D-glucosidic linkages in α -1,3- glucans, a major component of fungi cell wall (Simmons, 1994; Hoj and Fincher, 1995). So that cell lysis and cell death occur as a result of hydrolysis of glucans present in the cell wall of fungi.

**2.11.2. Chitinases (PR3)**

Most of Chitinase are having molecular mass in the range of 15 kda and 43 kDa. Chitinase can be isolated from *Cicer arietinum* (chickpea) (Saikia *et al.*, 2005), *Cucumis sativus* (cucumber), *Hordeum vulgare* (barley) (Kirubakaran and Sakthivel, 2006) Chitinase and α -1,3-Glucanase are differentially regulated by Wounding, Methyl Jasmonate, Ethylene, and Gibberellin. Wounding and methyl jasmonate induces gene *chi*

*9* for chitinases expression in the tomato seeds (Wu and Bradford, 2003). In some study, it is also found that chitinase gene are also expressed in response to stress like cold up to -2 to -5°c (Yeh *et al.*, 2000). These Chitinases have significant antifungal activities against plant pathogenic fungi like *Alternaria* sp. For grain discoloration of rice, *Bipolaris oryzae* for brown spot of rice, *Rhizoctonia solani* for sheath blight of rice (Chu and Ng 2005; Saikia *et al.*, 2005; Kirubakaran and Sakthivel, 2006).

## 2.12. Transgenic rice plants expressing PR-proteins

### 2.12.1. Chitinase genes (PR-3 family)

Rice was the first cereal to be transformed with genes for PR-proteins. Perhaps because of the ease with which it can be transformed (relative to other cereals), rice also ranks first among cereals for the number of different PR-protein genes that have been used to obtain transgenic plants. A chitinase gene, chi11, isolated from a rice genomic library (Huang *et al.*, 1991) was placed under the control of a camv 35S promoter and used for PEG-mediated transformation of rice protoplasts (Lin *et al.*, 1995). The effect of the added expression of the transgenic chitinase Chi-11 (in addition to the pathogen-inducible chitinases) on the resistance of transgenic plants to sheath blight was investigated by challenging control and transgenic plants with *R. Solani* (Lin *et al.*, 1995). Datta and Muthukrishn, (1994) also obtained transgenic plants expressing a pathogen-inducible rice chitinase gene, RC-7, by biolistic transformation of several elite lines of rice. Rice chitinase genes also have been introduced into other plants with beneficial effects against some pathogens (Datta *et al.*, 1999). Such as transgenic cucumber plants overexpressing the cht-2 gene had a significantly higher resistance to gray mold (Tabei *et al.*, 1998) compared to controls.

### 2.12.2. Proteinase inhibitor genes (PR-6 family)

A gene for a cowpea trypsin inhibitor (cpti) under the control of a rice actin 1 promoter was used to obtain transgenic rice plants by PEG-mediated transformation of protoplasts of a Japonica rice, Taipei 309 (Xu *et al.*, 1994). Some of the regenerated plants were found to be resistant to glufosinate and to express the cpti infairly large

amounts (1.3 per cent of total soluble protein) in leaf and stem tissues. A potato proteinase inhibitor gene (pin II) with its native wound-inducible promoter and a rice actin intron were introduced into Japonica rice by a biolistic procedure, and transgenic plants with high level of expression were identified (Duan *et al.*, 1996). The transgene was shown to be inherited stably over five generations.

**2.13. Induced systemic resistance (ISR) against pathogen**

Resistance to plant disease is often specific and metabolites and receptors contributing to this specificity may have specific structures. However, simple, structurally-unrelated compounds induce systemic resistance in unrelated plants to diverse pathogens including fungi, bacteria and viruses. Both resistance and induced systemic resistance (ISR) are associated with the rapid accumulation of the same structurally unrelated putative defense compounds that have diverse functions. It has been suggested that cultivar (race)-specific resistance is initiated by the specific interaction of a pathogen product (or pathogen induced product) and a plant receptor. However, restricted infection by pathogens can result in ISR and many different compounds can cause ISR. It is thus evident that there are both specific and non-specific routes to the master switch for ISR and there may be more than one master switch. Are reactive oxygen species and free radicals regulating the master switch (es) via both routes. It is also evident there are many switches, other than the master switch. Adding to the complexity of resistance and ISR are the observations that different compounds and pathways may mediate different biochemical resistances. Activation of one of the pathways may antagonize or enhance the activation or effectiveness of another (Kuc, 1995b).

Local infection by single strains of pathogenic bacteria, fungi or viruses can induce plants to develop a plant-wide resistance against future attack by a variety of microbial pathogens. In this phenomenon, called induced systemic resistance (ISR) salicylic acid is involved in this pathway (Hammerschmidt and Smith-Becker 1999; Cameron 2000; Matraux, 1990), but it is probably not the translocated signal, and if it is, then it is probably not the only signal (Hammerschmidt and Kuc. 1995). The systemically translocated signal induces the expression of a broad-spectrum, long-lasting

immunity against further pathogen infection in both infected and non-infected plant parts, called systemic acquired resistance (SAR) (Ross, 1961) or induced systemic resistance (ISR).

ISR is potentiated by plant growth-promoting rhizobacteria (PGPR), of which the best characterized are several species of *Pseudomonas* that cause no visible damage to the plant's root system. Unlike SAR, ISR does not involve the accumulation of pathogenesis-related proteins or salicylic acid (Pieterse *et al.*, 1996), but instead, relies on pathways regulated by jasmonate and ethylene (Knoester *et al.*, 1999; Yan *et al.*, 2002).

The classic form of SAR can be triggered by exposing plant to virulent, avirulent, and nonpathogenic microbes, or artificially with chemicals such as salicylic,2,6-dichloro-isonicotinic acid (INA) or benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) ( Sticher *et al.*, 1997). Depending on the plant and elicitor, a set period of time is required for establishment of SAR that corresponds to the time required for the coordinated accumulation of pathogenesis-related proteins (and transcripts) and salicylic acid throughout the plant (Cameron *et al.*, 1994; Ward *et al.*, 1991)

It is important to realize that SAR and ISR, as just defined, are probably only two outcomes out of an array of possibilities. It is likely that other forms of induced resistance exist that vary in their reliance on salicylic acid, ethylene, and jasmonate and other as yet discovered plant regulators. However, it is the availability of chemical inducers of SAR, such as BTH, and the characterization of numerous PGPR strains, that makes the applied use of induced resistance in conventional agriculture a reality.

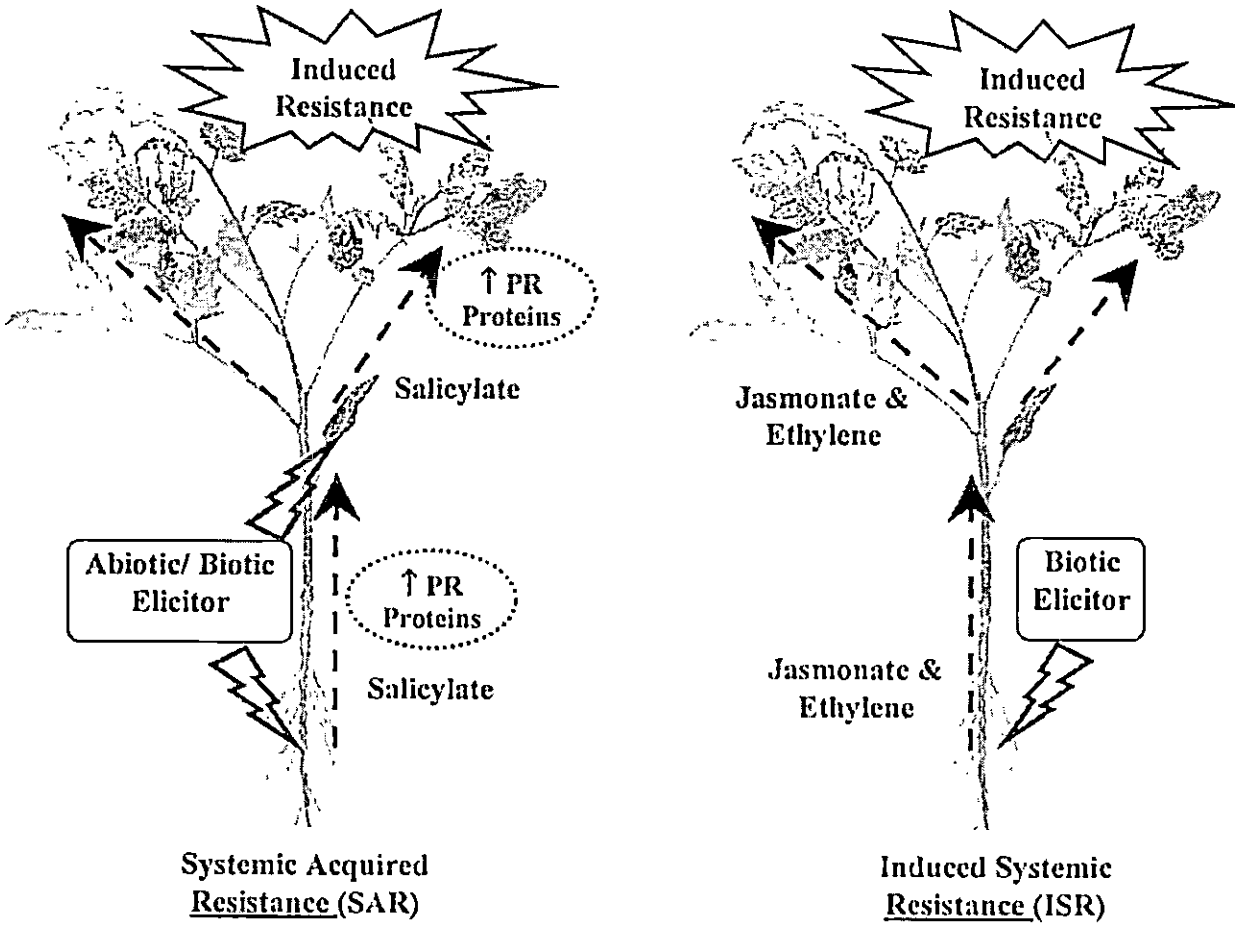



**Fig. A pictorial comparison of the two best characterized forms of induced resistance in plants, both which lead to similar phenotypic responses.**

### 2.13.1. Efficacy among Monocot

Gorlach *et al.* (1996) reported successful control of powdery mildew, Septoria leaf spot (caused by *Septoria* spp.), and leaf rust (caused by *P. Recondita* ) of wheat in field trials. BTH was also effective against downy mildew of maize (caused by *Peronoscleropora sorghi*) in the field when applied as a seed treatment (Morris *et al.*, 1998). Stadnik and Buchenauer (1999a, 1999b) reported success in field experiments with single applications of BTH for controlling powdery mildew of wheat, but had mixed results against Septoria leaf blotch (caused by *Septoria tritici* ).

### 2.13.2. Efficacy among Leguminous Crops

Limited field experiments involving the effects of INA and BTH on diseases of legumes have been reported (Dann and Deverall, 1996; Dann *et al.*, 1998). Reduced densities of uredinia of the rust fungus, *Uromyces appendiculatus* Unger, on trifoliolates of common were obtained when INA was applied at least 7 d before inoculation, but not at 2 h before inoculation (Dann and Deverall, 1996). An additional application of INA during pod-set did not improve resistance of common bean plants to *U. Appendiculatus*, as opposed to a single application to the first trifoliolate (Dann and Deverall, 1996).

### 2.13.3. Efficacy on Fruit Trees

Of particular interest is the reported control of several diseases of apple (*Malus domestica* Borkh.) and Japanese pear (*Pyrus pyrifolia* Nakai) with BTH in field strains was trials (Ishii *et al.*, 1999; Maxson-Stein *et al.*, 2002). The ability of BTH to inhibit the extension of fire blight cankers was directly proportional to the application rate. BTH application induced the expression of several pathogenesis-related genes (*PR-1*, *PR-2*, and *PR-8*) in greenhouse grown apple seedlings (Maxson-Stein *et al.*, 2002). Other greenhouse studies have found promise for the use of BTH in citrus against scab (caused by *Elsinoe" fawcettii* Bitancourt and Jenk.),melanose (caused by *Diaporthe citri* F.A. Wolf), and Alternaria brown spot [caused by *Alternaria alternate* (Fr.:Fr) Keissl.] (Agostini *et al.*, 2003)

## 2.14. Induced systemic resistance (lsr) in rice for bacterial blight

Wu.Q. *et al.* (2011) investigated pathogenesis-related (PR) protein expression in interactions between rice plants with bacterial blight resistant gene Xa21 and Xanthomonas oryzae pv. Oryzae (Xoo), the causal agent of bacterial blight. Three PR genes, osprla, osprlb, and osprl0a, were cloned and the fusion proteins were expressed in *E. Coli* to generate polyclonal antibodies. The proteins isolated at 0, 12, 24, 36, 48, 60, 72, and 144 h post-inoculation (hpi) from rice leaves inoculated with incompatible Xoo strains were analyzed using Western blotting. Significant inductions were observed for all three PR proteins in the late stages of inoculation. They also compared the expression of

PR proteins among four different modalities: (i) incompatible interactions (R); (ii) compatible interactions in the absence of the Rgene (SR -); (iii) compatible interactions in the absence of the avirulence factor in Xoo (savr -); (iv) mock inoculated control treatments (M). Results showed that the three PR proteins are enhanced in the R, SR -, and savr - interactions more than in the control treatments. The induction of PR proteins in the R interaction is the highest, suggesting that these proteins are involved in the defense mechanism.

Mohan Babu *et al.* (2003) found the role of the plant defence activator, acibenzolar-S-methyl (ASM), in inducing resistance in rice against bacterial leaf blight caused by *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*) was studied. Application of ASM induced resistance in rice to infection by *Xoo*. When the pathogen was clip-inoculated to the rice plants, it caused bacterial leaf blight symptoms in the untreated control. However, in the rice plants pretreated with ASM, infection was significantly reduced. Induced systemic resistance was found to persist for up to 3 days in the pretreated rice plants. Increased phenolic content and accumulation of pathogenesis-related (PR) proteins, *viz.* Chitinase, β-1, 3-glucanase and thaumatin-like protein (TLP; PR 5) were observed in rice plants pretreated with ASM followed by inoculation with *Xoo*. Immunoblot analysis using rice TLP and tobacco chitinase antiserum revealed rapid induction and over-expression of 25 and 35 kda TLP and chitinase, respectively, in rice in response to pretreatment with ASM followed by *Xoo* inoculation. Based on these experiments, it is evident that induction of disease resistance in rice was accelerated following treatment with ASM.

### 2.14.1. Defense Pathways

In response to microbial attack, plants activate a complex series of responses that lead to the local and systemic induction of a broad spectrum of antimicrobial defenses (Hammond-Kosack, and Jones, 1996). Much progress has been made in understanding the mechanisms by which plants detect and defend themselves against microbial attack. Recent advances have been made in several areas. These include the cloning and characterization of plant disease resistance genes that govern the recognition of specific

pathogen strains (Dangl and Jones, 2001; Staskawicz, 2001), the identification of components involved in the signal transduction pathways coupling pathogen recognition to the activation of defense responses (Glazebrook, 2001; Feys and Parker, 2000.) And the demonstration that three endogenous plant signaling molecules, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), are involved in plant defense (Dong, 1998; Thomma, *et al.*, 2001) .

These signaling molecules are involved in what appear to be two major pathogen defense signaling pathways: an SA-dependent pathway and an SA-independent pathway that involves JA and ET. These pathways do not function independently, but rather influence each other through a complex network of regulatory interactions. A greater understanding of the SA, JA and ET signaling pathways and of how they modulate each other should provide insight into the mechanisms underlying the activation and regulation of defense responses. This may also provide insight into strategies that are used by plant pathogens to alter host defense responses, and thus to promote pathogen virulence and disease production.

**2.15. The importance of proteomics**

Proteomics has been defined as "the systematic analysis of the protein population in a tissue, cell, or subcellular compartment." Proteomics is a relatively recent field; the term was coined in 1994. Studying proteins generates insight on how they affect cell processes. Conversely, this study also investigates how proteins themselves are affected by cell processes or the external environment.

Proteins provide intricate control of cellular machinery, and are in many cases components of that same machinery (Graves and Haystead, 2002). They serve a variety of functions within the cell, and there are thousands of distinct proteins and peptides in almost every organism. This great variety comes from a phenomenon known as alternative splicing, in which a particular gene in a cell's DNA can create multiple protein types, based on the demands of the cell at a given time.

The goal of proteomics is to analyze the varying proteomes of an organism at different times, in order to highlight differences between them. Put more simply, proteomics analyzes the structure and function of biological systems (Van Wijk, 2001). The realization of this goal is difficult; both purification and identification of proteins in any organism can be hindered by a multitude of biological and environmental factors (Weaver and Robert, 2002).

Initially, proteomics focused on the generation of protein maps using two-dimensional polyacryl amide gel electrophoresis. The field has since expanded to include not only protein expression profiling, but the analysis of post-translational modifications and protein-protein interactions. Protein expression, or the quantitative measurement of the global levels of proteins, may still be done with two-dimensional gels, however, mass spectrometry has been incorporated to increase sensitivity, specificity and to provide results in a high-throughput format. A variety of platforms are available to conduct protein expression studies and this site provides links to these resources.

The study of protein-protein interactions has been revolutionized by the development of protein microarrays. Analagous to DNA microarrays, these biochips are printed with antibodies or proteins and probed with a complex protein mixture. The intenisty or indentity of the resulting protein-protein interactions may be detected by fluorescence imaging or mass spectrometry. Other protein capture methods may be used in place of arrays, including the yeast two-hybrid system or the isolation of proteins/protein complexes by affinity chromatography or other separation techniques.

## 2.16. Proteomics for Crop Improvement

In 1999, the first review of plant proteomics was published and extensively discussed the plant proteomics literature before 1999 (Thiellement *et al.*, 1999). Most of these studies did not involve MS and therefore were limited to the comparison of expression levels without actual identification of the proteins. In a few cases, limited sets of proteins were identified through Edman sequencing. Many of these studies were focused on the use of 2-DE patterns to identify possible markers for different genotypes and phenotypes and phylogenetic relationships.

Around 1996, a group of European scientists formed a European Union-supported consortium to study the proteome of the plasma membrane of tobacco and *Arabidopsis*. This resulted in a number of fairly methodological studies, the construction of 2-DE reference maps (Rouquie *et al.*, 1997; Santoni *et al.*, 1999). A number of plasma membrane-specific proteins were identified and most of these papers highlighted the failure to use 2-DE gels for reproducible and complete mapping of membrane proteins.

The pattern of protein synthesis during hypoxic acclimation and anoxia in maize roots was analyzed by incorporation of$^{35}$s-Met combined with 2-DE and MS (Chang *et al.*, 2000). This work showed that protein synthesis during acclimation, but not during subsequent anoxia, is crucial for acclimation. This work shows some of the potential and difficulties of proteomics to study up-and down-regulation of protein expression.

The chloroplast is predicted to contain maximally 2,500 to 3,000 proteins expressed in a wide dynamic range; these studies demonstrate that further purification of these proteins into sub-proteomes and protein complexes is an effective strategy to obtain a more in depth insight (Van Wijk, 2001).

Ferro *et al,* (2000) used organic solvent fractionation followed by SDS-PAGE to purify and identify integral chloroplast envelope proteins . The propensity of hydrophobic proteins to partition in chloroform/methanol mixtures was directly correlated to the ratio between molecular mass and the number of putative transmembrane regions.

The proteins of the 30S and 50S ribosomal subunits in spinach chloroplasts were identified by a combination of 2-DE, chromatography, MS, and Edman sequencing (Yamaguchi and Subramanian, 2000; Yamaguchi *et al.*, 2000). It was concluded that the spinach plastid ribosome comprises 59 proteins, of which 53 are *Escherichia coli* orthologues and six are non-ribosomal plastid-specific proteins (PSRP-1 to PSRP-6).

# Materials and Methods

# 3. MATERIALS AND METHODS

The study on 'proteome analysis of induced systemic resistance mediated by plant growth promoting rhizobacteria (PGPR) in rice for biotic stress' was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), IT-BT Complex, College of Horticulture, Vellanikkara during August, 2011 to July, 2012. Materials used and methodologies adopted for the studies are described in this chapter.

## 3.1. Plant material and *Pseudomonas fluorescens*

Jyothi (PTB -39), a popular rice variety of Kerala and susceptible to blight and BPH was used as the experimental material. The pot culture experiment was carried out by direct sowing. The seed material was obtained from the department of Plant Breeding and Genetics, College of Horticulture, Kerala Agricultural University, Vellanikkara. *Pseudomonas fluorescens* strain *Pf1* (KAU culture) was obtained from the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture.

### 3.1.2. Chemicals, glassware and plastic ware

All the chemicals used in the study were of good quality (AR/GR grade) obtained from various firms such as Merck, Sisco Research Laboratories, Himedia and Sigma.

### 3.1.3. Laboratory equipments

Instruments available at CPBMB were utilized for the work Bioinformatics softwares were accessed from Distributed Information Centre (DIC).

## 3.2. Methods

### 3.2.1. Treatment to the plants

The plants were given two treatments. Plant without any treatment served as control. In second treatment *Pseudomonas fluorescens* was applied. Fifteen pots were maintained for each treatment and seven plants were maintained in each pot. The design used was completely randomized block design. Plant were given two application of

*Pseudomonas fluorescens* ie, seed treatment and foliar spray described the details are in (3.2.2 and 3.2.3.) water logged condition was maintained by sealing the whole at side of pot (Plate 1).

### 3.2.2. Seed treatment with *Pseudomonas fluorescens*

Rice seeds were soaked in sterilized distilled water containing talc based formulation (10g/kg of seed) of *Pseudomonas fluorescens*. After 24 h, the suspension was drained off and the seeds were dried under shade for 30min and used for sowing (Vidhyasekaran *et al.*, 1997a).

### 3.2.3. Foliar spray with *Pseudomonas fluorescens*

The talc based product was dissolved in water (20 g/L) to get 2 per cent concentration was sprayed 35 days after sowing.

## 3.3. Efficacy of *Pseudomonas fluorescens* on sheath blight disease

### 3.3.1. Isolation and culturing of pathogen

Rice sheath blight pathogen *Rhizoctonia solani* was isolated from infected leaf sheath. Portion of leaf sheath showing typical lesions were cut into small bits and surface sterilized with 0.1 per cent sodium pyrochlorite and plated on Potato Dextrose Agar (PDA) medium (Riker and Riker,1933). Axenic culture of pathogen was obtained by single hyphal tip technique (Rangaswami, 1972) and maintained in PDA slant at laboratory temperature to use further for challenging (Plate 2a).

### 3.3.2. Challenge inoculation with *R. solani*

Fourty five days old plants were inoculated with immature white sclerotia from four days old culture of *R. solani*. The immature sclerotia were placed between the leaf sheath and stem without any injury and wrapped with moisten cotton. Inoculated plants were covered with polythene cover to maintain humidity (Plate 2b). The inoculation was carried out on both *Pf* treated and non treated plants. Five pots were kept for each treatment. Sheath blight intensity was calculated seven days after inoculation, graded on a


**Plate 1. Experimental layout**

**Plate 2a. *Rhizoctonia Solani* Culture**

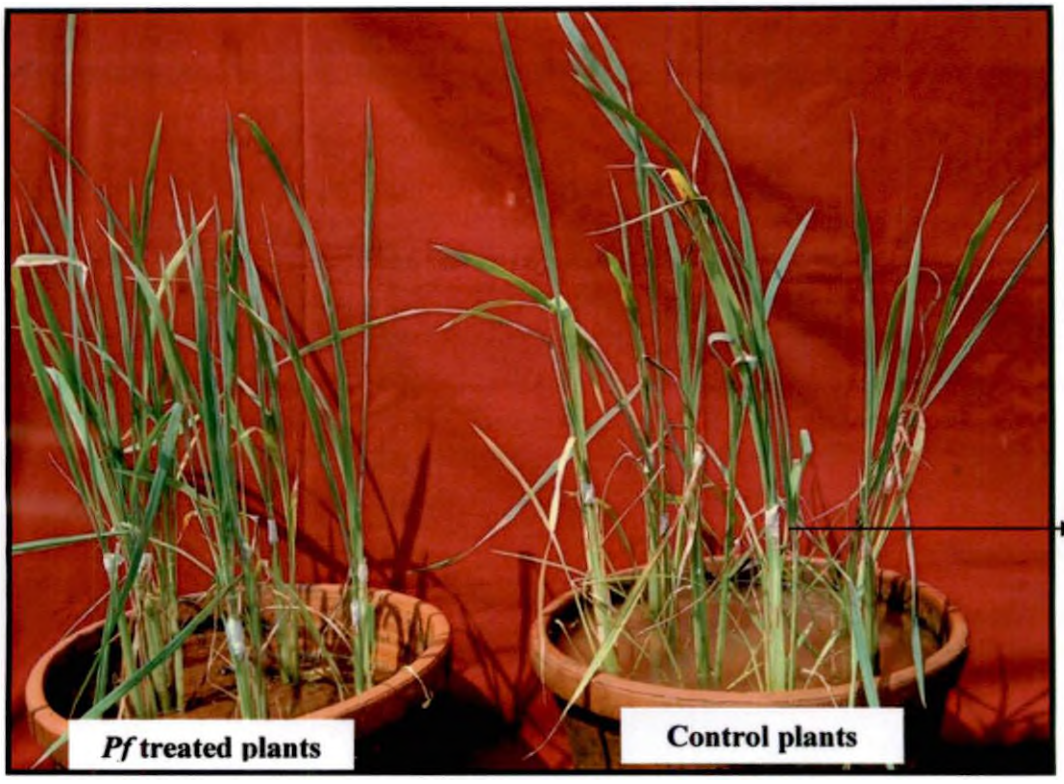



**Plate 2b. Plants challenged with *R. solani***

0-9 scale of Standard Evaluation System for rice, IRRI (1980) and compared on the basis of per cent disease incidence using fallowing formula, suggested by Wheeler (1969).

$$\text{Per cent Disease Index} = \frac{\text{Sum of all numerical rating}}{\text{Total number of tillers observed } \mathbf{X} \text{ Maximum disease grade}} \text{ X } 100$$

Whereas the grade was given on the basis of the description

| Grade | Description |
|---|---|
| 0 | No incidence |
| 1 | Lesion limited to lower ¼ of leaf sheath area |
| 3 | Lesion present in ½ lower of leaf sheath area |
| 5 | Lesion present on more than ½ of leaf sheath area, slight infection on lower one or two leaves |
| 7 | Lesion present on more than ¾ of leaf sheath, sever infection on lower leaves and slight infection on upper leaves |
| 9 | Lesion reaching top of tillers, sever infection on all leaves and few plants killed |

### 3.4. Efficacy of fluorescent pseudomonad bioformulations against BPH insect

#### 3.4.1. Mass rearing of brown planthopper (*N. lugens*)

The method outlined Medrano and Heinrich (1985) was followed in mass culturing of BPH. The rice seedlings were raised in earthen pots and kept partially immersed in plastic tray containing water. These trays were kept in insect rearing cages (3×2.5×2.75m) with netting. Gravid females of brown planthopper (BPH) were collected from the fields of KVK Thrissur and Regional Agricultural Research Station, Patambi and released on individual plants kept inside the rearing cages for oviposition. Nymph emerged a week after oviposition and damaged plants due to feeding of BPH were

periodically replaced with fresh potted plants. The plants were made free of spiders, mirid bugs and ants for effective culturing of BPH (Plate 3).

### 3.4.2. Challenge inoculation of BPH

Five pots each of control and *Pseudomonas fluorescens* treated were used for assessing the efficiency of pseudomonas treatments on rice plants against BPH, fourth and fifth instar nymphs of brown planthopper (BPH) carefully collected from the cage by using aspirator starved for 5 h and released at the rate of 10 numbers per pot on to sheath of pseudomonas treatments forty five days old rice plants and covered with mylar film and allowed to feed for seven days (Plate 4a and 4b). Per cent mortality and damage caused by BPH was calculated by using standard scale.

$$\text{Per cent mortality} = \frac{\text{Total number of BPH released} - \text{Number of BPH alive}}{\text{Total number of BPH released}} \times 100$$

Damage rating was done as per Standard Evaluation System for rice, IRRI (2002)

| Scale | Description |
|---|---|
| 0 | No Damage |
| 1 | Very slight damage |
| 3 | 1st and 2nd leaves of most plant partially yellowing |
| 5 | Pronounced yellowing and stunting or about 10 to 25 per cent of the plants wilting or dead and remaining plants severely stunted or dying |
| 7 | Pronounced yellowing and stunting on more than half of the plants |
| 9 | All plants dead |

**Plate 3. Mass rearing of BPH**

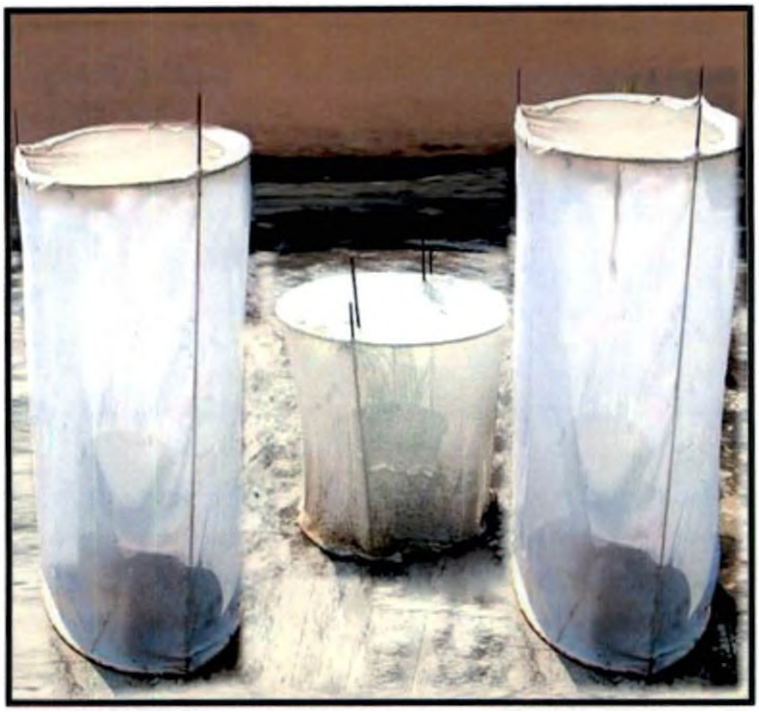


**Plate 4a. Control plants challenged with insect (BPH)**

**Plate 4b. *Pf* treated plants challenged with insect (BPH)**

### 3.5.3. Measurements on biometric parameters

The observations were made on shoot length, root length, number of tillers, fresh weight and dry weight for biometric analysis in control and *Pf* treated plants after 30 days of sowing. Statistical analysis was carried out using T-test.

## 3.6. Analysis of proteome

### 3.6.1. Plant materials

Forty-five day old plants were inoculated with pathogen (*R.solani*) and insect (brown planthopper). After 24h of inoculation, observations were made on pest/disease development based on the symptoms. Leaf sheaths were collected in liquid nitrogen from the treatments as per given below for protein extraction.

### 3.6.2. Treatment details

**T1**- PGPR treatment and challenged with *Rhizoctonia solani.*

**T2**- PGPR treatment and challenged with BPH.

**T3**- Inoculation with *Rhizoctonia solani* alone.

**T4**- Inoculation with BPH alone.

**T5**- PGPR alone.

**T6**- Absolute control.

## 3.7. Challenge inoculation with insect and pathogen

Challenge inoculation was carried out as in **3.2.2** and **3.3.2.**

## 3.8. Assay of defense- related enzymes

### 3.8.1 Sample collection

Samples were collected from individual treatments to study the induction of defense enzyme in response to pathogen and pest attack in rice plant. Leaf and sheath

tissues from each treatment underlined in 3.6.1. were collected at 0, 6, 24, 48, 72, and 96 hours intervals after inoculation.

### 3.8.2 Protein extraction

Leaf and sheath tissues were collected from each treatment at different time interval and immediately homogenized with liquid nitrogen. One gram of powdered sample was extracted with 2ml of 0.1 m Sodium citrate buffer (pH 5.0) at $4^0$ C. The homogenate was centrifuged for 20 min at 10,000 rpm. The supernatants were stored at -$20^0$C until further use for protein analysis and enzyme assays (Lawrence *et al*., 1996)

### 3.8.3 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 up to 100 ml with distilled water. A sample of 50 µl was added to 950 µl of dye solution and the mixture was incubated for 5 min at room temperature. The absorbance was recorded at 959 nm in spectrophotometer. Bovine serum albumin was used as the standard.

## 3.9. Proteome analysis by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

PAGE was performed in 12 per cent (w/v) slab gels containing SDS according to Laemimli (1970). Proteins were separated by SDS-PAGE in Bio-Rad (Hercules, CA, USA) big gel electrophoresis unit with 12 per cent resolving and 4 per cent stacking gel. The plates were assembled in gel casting apparatus. Then poured the resolving gel (30 per cent acrylamide, 1.0 M tris Hcl 8.8, 10 per cent SDS, 10 per cent APS and TEMED 0.01 per cent) and overlaid with water. Allowed to polymerise for 1-2h and discarded the water layer. Poured the stacking gel solution and allowed to polymerise after placing comb. Removed the comb and assembled the plates in unit. Poured tank buffer (tris-glycine- SDS buffer). Protein samples 150µg were diluted with equal volumes of 2X sample buffer and place it in a boiling water bath for 90s and then kept in ice before loading. Each well was loaded with protein samples from different treatment and the gels

were electrophoresed for 2h at a constant current of 20 mA. Electrophoresis was carried out using running buffer (Tris, Glycine, SDS pH 8.3). After electrophoresis, gels were stained with Coomassie blue stain solution (0.025 per cent Coomasie brillant blue R250, 40 per cent methanol, 7 per cent acetic acid and distilled water) then destained with destain solution I (40 per cent methanol, 7 per cent acetic acid and double distilled water) and destain solution II (7 per cent acetic acid, 5 per cent methanol and double distilled water). The stained gel was documented in gel documenting unit.

### 3.10. Assay of peroxidases

Leaf sheath samples (1 g) were homogenized in 2 ml of 0.1M phosphate buffer, pH 7.0 at $4^0$C. The homogenate was centrifuged at 16000 g at $4^0$C for 15 min and the supernatant was used as enzyme source. 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 per cent $H_2O_2$. The reaction mixture was incubated at room temperature (28 ± 2°C). The changes in absorbance at 420 nm were recorded at 30 sec intervals for 3 min. The enzyme activity was expressed as changes in the absorbance $min^{-1}mg^{-1}$protein (Hammerschmidt *et al.*, 1982).

### 3.11. Protein profiling by Western blot

For western blot analysis polyclonal antibody for chitinase was developed by using Ausubel *et al.*, 1990 protocol.

#### 3.11.1. Development of polyclonal antibody

##### 3.11.1.1. Antigen preparation

First *Trichoderma harzianum* was grown on oat will agar medium. Culture was filtered. The filtrate contained extracellular enzymes. Enzyme was precipitated by using 90 per cent $NH_4SO_4$ and culture was saturated in ice condition. It was kept overnight for incubation at $4^0$c. Culture was centrifuged at 8000 rpm for 15 minutes. Obtained pallet was crude protein which was dissolved in $PO_4$ buffer. Crude protein was dialyzed using semi permeable membrane.

### 3.11.1.2. Antigen purification- dialysis of protein

The dialysis tube (width - 29.31 mm, diameter - 17.5 mm, capacity- 2.41 ml/cm) was cut into appropriate length and placed in 2 L beaker containing 1 L of preheated D/W. Then it was drained and boiled in 1 L solution of 2 per cent sodium carbonate and 1mM EDTA (pH-5.0) for 30 min. It was drained again and rinsed twice in D/W followed by boiling in D/W for 30 min. It was drained and rinsed twice in D/W using a sterilized forceps and again rinsed in 25 per cent ethanol. After draining the treated tubing was stored in 25 per cent Ethanol at $4^0$ C until use.

Just before use, the treated dialysis tube was washed in D/W. Leaving an inch or two from one of the open end, dialysis tubing-specific closure was used to close that end and the part was cut off from the remaining tubing. The precipitated protein sample was pipette out in to the tubing and the other end was closed off with another closure. It was then inserted in a large beaker containing water and the volume of water was 100 times of that of the protein sample. The beaker was kept in a shaking incubator with temperature control, set at $4^0$C and stirred for 1.5 hrs. Water was then discarded and beaker was filled with same amount of water and dialyzed for overnight. Next day tubing was removed from beaker and one end was carefully opened. The protein was pipetted out and stored at $-20^0$C.

### 3.11.1.3. Collection of pre immune serum

The thigh vein was selected in rabbit. The hair was removed from particular area. Xylene was applied to dialate the vein. A minute cut was made in vein. Blood was collected in eppendorf tube. Then the portion was swabbed with 70 per cent ethanol to remove xylene. The collected blood was kept at $37^0$C for 1 h where the serum was not coagulated. Then it was kept at $4^0$C for overnight. On next day serum was taken and subjected to 10000 rpm for 5-10 minutes to obtain serum. 0.01 per cent sodium azide was added to prevent from bacteria and fungi.

### 3.11.1.4. Antigen injection in rabbit

#### 3.11.1.4.1. Immunization schedule

0-day- collection of pre immunized serum

5-day- 1st injection (adjuvant (complete) + protein of interest)

15-20-days- Booster dose 1 adjuvant + protein of interest (incomplete without bacteria, only mineral oil)

30-days- Booster dose 2

60-days- Booster dose 3

1 ml of complete adjuvant, which contain mineral oil, surfactant and *Mycobacteriaum*, was used. 1 ml of antigen was taken. It was then mixed with adjuvant by continuous stirring (it was stirred until the drop of emulsion was not dispersed in water). Emulsion was the final product that came by mixing adjuvant and antigen (protein of interest). 20µl of the emulsion was taken in syringe and injected to rabbit in its thigh region.

### 3.11.1.5. Booster dose for rabbit

On the 15th day of antigen injection the 1st booster dose was given. Freunds incomplete adjuvant was mixed in equal quantity with the protein of interest (incomplete adjuvant in it, without heat killed bacteria, only mineral oil). As the mineral oil was highly viscous it was thoroughly mixed with antigen to avoid the malfunctioning of the animal system.

On the 30th day second booster dose was given.

On the 15th day of second booster dose, rabbit was bleed for antibody production.

### 3.11.1.6. Collection of blood from immunized animal and antibody purification

Blood sample (1ml) was collected from immunized rabbit using sterile syringes. Sterile eppendorf tubes were labeled properly and left undisturbed until it clotted at room

temperature up to one hour. Clot was removed gently by using toothpick and kept overnight at $4^0$C. After overnight incubation the clear liquid was carefully collected and spun at 10000 rpm at $4^0$C for 5-10 minutes. The top straw colored suspension was collected in sterile eppendorf and stored at $-20^0$C.

### 3.11.2. Ouchterlony double-immuno diffusion to detect the antibody presence

Ouchterlony double immuno diffusion was carried out to detect the antibody in pre and post immune serum of rabbit.1.2 per cent agarose (0.3 g/25ml) was prepared in 1x assay buffer by boiling to dissolve the agarose completely. It was then cooled to 55-$60^0$C, 4ml of solution was poured for one plate on to grease free glass plates placed on a horizontal surface. Gel was allowed to set for 30 minutes. Wells were punched by keeping the glass plate on the template. Wells were filled with 10 micro litters of the chitinase antiserum and the corresponding antigens. Three different concentrations of antibody were used via, 1:3000, 1:1000 and 1:500. Glass plate was kept in moist chamber overnight at $37^0$C. After incubation, observed for opaque precipitin lines between the antigen and antisera wells.

### 3.11.3. Western blot analysis

PAGE of proteins was performed in 12 per cent (w/v) slab gels containing SDS according to Laemmli (1970). Western blotting, incubation with antisera and immunological detection were performed according to method of Laemmli (1970) with suitable modifications. Chitinase antiserum developed in rabbit was used. Proteins (150 µg) were separated by SDS- PAGE in Bio-Rad (Hercules, CA, USA) big gel electrophoresis unit with 12 per cent resolving and 4 per cent stacking gel. The gels were elctrophoresed for 4h at a consatant current of 20 mA. After electrophoresis, the resolved proteins were electrotransferred to a polyvinylidine difluride (PVDF) membrane (Bio-Rad Hercules, CA, USA) for 30 min at 140 mA with semi-dry transblot apparatus (Bio-Rad Hercules, CA, USA) in accordance with the manufacterer's instructions. The membrane was then blocked with Tris-buffered saline Tween (TBS; 10 mM Tris-HCl, pH 7.9, 140 mM NaCl, 0.05 per cent (v/v) Tween-20) supplemented with 2.5 per cent (w/v) gelatin for overnight at room temperature ($28\pm 2^0$C), then washed three times (5

min each) with TBST. The membrane was then incubated at room temperature in TBST containing primary antibody at 1: 500 dilution shaker for a period of 3h. Antiserum raised against a chitinase was used as primary antibody. The membrane was washed five times (5 min each) with TBST. It was then inocubated in TBST containing horse radish peroxidase ( HRP) conjugated goat-anti rabbit IgG ( Bio- Rad) at 1:1000 dilution for 3h, washed three times (5 min each) with TBST and two times with TBS. Binding of the secondary antibody was detected by reaction of the antibody-HRP-conjugate with freshly prepared substrate solueion consisting of 15 ml of 30 per cent H2O2, 5 ml of 0.3 per cent (w/v) 4-chloro-1-napthol (Bio-Rad) in methanol, and 25 ml of TBS for 3 to 5 min. Apparent molecular mass of proteins was determined by comparison with standards.

### 3.12. Sequencing of protein bands

The up regulated and differentially expressed protein bands were sequenced by using MASCOT /MS peptide search engine in Sandor Proteomics, Hyderabad.

# Results

# 4. RESULTS

The study entitled 'proteome analysis of induced systemic resistance mediated by plant growth promoting rhizobacteria (PGPR) in rice for biotic stress was carry out at the Centre for Plant Biotechnology and Molecular Biology(CPBMB), IT-BT Complex, College of Horticulture, Vellanikkara, during August, 2011 to July, 2012. The result obtained in present study is presented below.

## 4.1. Pathogenicity test for *R.solani*

Rice plants when inoculated with *R. solani* produced typical symptoms of sheath blight disease within seven days of inoculation. Initial symptoms were minute specks and small lesions. Later the lesions were enlarged with greyish white centre and brown margins. Finally several lesions were coalesced and showed blighted symptoms. Infection started near the water line and it was spread upward to the upper leaf sheath and leaf blades. The entire plant was dried up at later stage.

The per cent disease intensity (PDI) was more on the control plants compared to that of *Pf* treated plants. The PDI of control plants was 47.33 per cent where as that of *Pf* primed plant was only 14.19 per cent as shown in table 1. This indicates that *Pf* induces resistance in rice plant for *R. solani* hence *Pf* primed plants showed low PDI (Plate 5).

## 4.2. Per cent mortality and damage by BPH

Feeding preference of BPH and the damage of BPH on rice plants was highly influenced by the application of *Pf.* Nearly 28 per cent mortality was observed in *Pf* treated plants. There was no mortality in control plants. (Table 2 and 3)

The damage caused due to BPH was rated by using 0-9 scale of Standard Evaluation System for rice, IRRI (2002). Observations were taken seven days after

A B C D

**1. *Pf* treated**

A B C D

**2. Control**

A: 24h after inoculation C: 72h after inoculation

B: 48h after inoculation D: 96h after inoculation

**Plate 5. Appearance of sheath blight symptoms on *Pf* treated and control plants at different time intervals after inoculation with *R. solani***

**Table1. Effect of *Pf* treatment on sheath blight intensity**

| | Per cent disease intensity | | | | | |
|---|---|---|---|---|---|---|
| **Treatments** | **R1** | **R2** | **R3** | **R4** | **R5** | **Mean** |
| *Pf*+ *R.solani* | 8.11 | 14.35 | 13.13 | 18.5 | 16.9 | 14.19 |
| Control+ *R.solani* | 38.8 | 47.08 | 52.38 | 46.03 | 52.38 | 47.33 |

**Table 2. Per cent mortality of BPH in *Pf* treated and control plants**

| Treatments | R1 | R2 | R3 | R4 | R5 | Mean |
|---|---|---|---|---|---|---|
| *Pf* + BPH | 30 | 20 | 30 | 40 | 20 | 28 |
| Control + BPH | 0 | 0 | 0 | 0 | 0 | 0 |

**Table 3. Damage scale rated by SES on control and *Pf* treated plants by BPH**

| Treatments | R1 | R2 | R3 | R4 | R5 | Mean |
|---|---|---|---|---|---|---|
| control + BPH | 4.15 | 3.9 | 4.8 | 3.8 | 4.6 | 4.52 |
| *Pf* + BPH | 0 | 0 | 0 | 0 | 0 | 0 |

release of insect. There was no damage on plants treated with *Pf* whereas control plants were fed by BPH.

The *Pf* treated rice plants showed high level of resistance to BPH attack. There was no damage on plants treated with *Pf* but control plants were susceptible.

**4.3. Measurement of biometric parameters**

The plant observations on shoot length, root length, number of tillers, fresh weight and dry weight were taken 30 days after sowing on both the control and treated plants. Statistical analysis was carried out using T- Test. The maximum shoot length, root length, fresh weight and dry weight were observed in *Pf* treated plants when compared to control plants. There was 4.08 per cent increase in shoot length, 28.01 per cent increase in root length, 13.14 per cent increase in number of tillers, 14.83 percent increase in fresh weight and 7.06 per cent increase in dry weight was observed in *Pf* primed plants over control plants. The results indicated the growth promotion effect of PGPR (Table 4 and Plate 6).

**4.4. Protein profiling by SDS page**

**4.4.1. Analysis of proteins from *Pf* treated and control rice plants challenged with *R. solani***

The protein banding pattern was studied in rice plants treated with *Pf* and challenged with *R. solani*. The banding pattern was predominant in *pseudomonas* treated plants challenged with *R. solani* than the uninoculated control plants. The total protein was collected at an interval of 6, 24, 48, 72 and 96 hours after treatment for SDS-PAGE analysis.


**Treatment details**

1 and 2- *Pf* treated plants

3 and 4- Control plants

**1. Plants with and without *Pf* (30 Days after sowing)**


**Treatment details**

1-4. Roots of control plants

5-8. Roots of *Pf* treated plants

**2. Enhanced root growth in rice seedlings treated with *Pseudomonas fluorescens***

**Plate 6. Enhanced growth promotion in rice seedlings treated with *Pseudomonas fluorescens***

**Table 4. Measurement on biometric parameters**

| Characters | *Pf* treated | Control | T value |
|---|---|---|---|
| Shoot length(cm) | 33.26 | 31.98 | 6.74** |
| Root length(cm) | 39.41 | 28.37 | 3.54** |
| Number of tillers | 3.50 | 3.04 | 3.73** |
| Fresh weight/plant(g) | 36.19 | 30.82 | 7.7** |
| Dry weight/plant(g) | 27.64 | 22.68 | 10.47** |

** Significant at 1% level

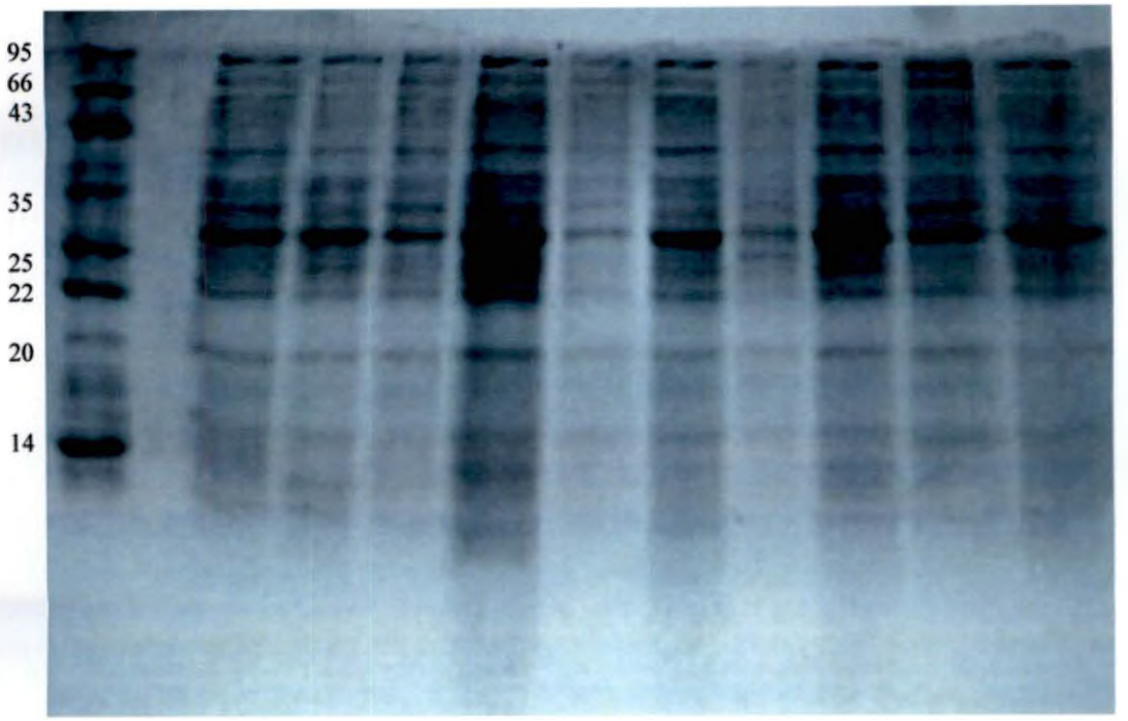
SDS-PAGE analysis of proteins from leaf extract of rice showed clear differences among *Pf* treated and control plants challenged with *R. solani.* At 6h after inoculation, 16 proteins were expressed in *Pf* treated plants and 12 in control plants. At 24, 48, and72h, proteins were up regulated in *Pf* treated plants unlike control plants. The banding pattern of proteins expressed at 96h after inoculation was same in both *Pf* treated and control plants (Plate 7).

### 4.4.2. Analysis of proteins from *Pf* treated and control rice plants challenged with BPH

The protein banding pattern was studied in rice plants treated with *Pf* after challenge inoculation with BPH insect. The banding pattern was predominant in *Pf* treated plants challenged with BPH than the uninoculated control plants.

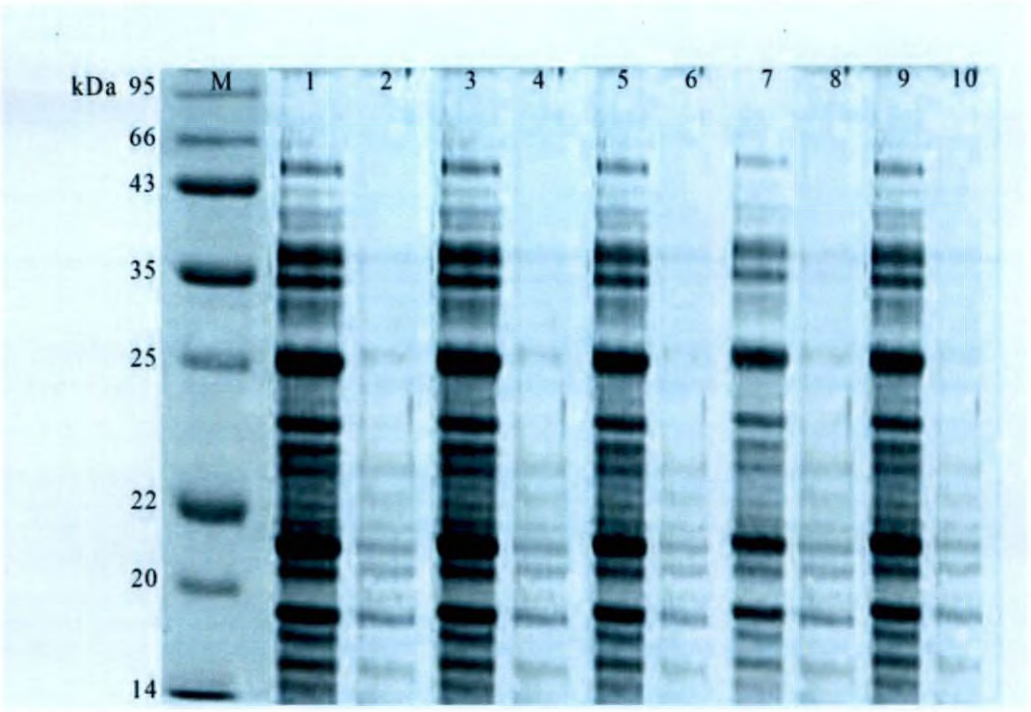
The total protein was collected at an interval of 6, 24, 48, 72 and 96 hours after treatment for analysis. The changes in the proteome of PGPR primed and nonprimed rice leaf tissue challenged with BPH were compared and analyzed (Plate 8).

SDS-PAGE analysis of proteins from leaf extract of rice showed clear differences among *Pf* treated and control plants challenged with BPH. At 6, 24, 48, 72 and 96h after challenge inoculation, all the proteins were up regulated in *Pf* treated plants over the control plants. There was expression of 25 proteins in all the *Pf* treated plants where as only 13 in control plants. There is up regulation and differential expression in *Pf* treated and control plants in all the treatments. The 19, 23 and 30 kDa proteins were differentially expressed in all *Pf* treated plants, as these proteins were specifically expressed in *Pf* treated plants and not in control plants (Plate 8).




M: Protein marker
1: 6h control + *R.solani*
2:6h *Pf* treated + *R.solani*
3: 24h control + *R.solani*
4: 24h *Pf* treated + *R.solani*
5: 48h control + *R.solani*
6: 48h *Pf* treated + *R.solani*
7: 72h control + *R.solani*
8: 72h *Pf* treated + *R.solani*
9: 96h control + *R.solani*
10: 96h *Pf* treated + *R.solani*

**Plate 7. SDS- PAGE analysis of proteins from plants treated with and without *Pf* challenged with pathogen**




M: Protein marker
1: 6h *Pf* treated + BPH
2: 6h Control + BPH
3: 24h *Pf* treated + BPH
4:24h Control + BPH
5: 48h *Pf* treated + BPH
6: 48h Control + BPH
7: 72h *Pf* treated + BPH
8: 72h Control + BPH
9: 96h *Pf* treated + BPH
10: 96h Control + BPH

**Plate 8. SDS-PAGE analysis of proteins from plants treated with and without *Pf* challenged with BPH**

### 4.5.1. Protein analyzed by MS MALDI-TOF

The profiling of proteins was studied by SDS-PAGE in *Pf* treated and control plants challenged with *R. solani* and BPH at different time intervals. Differentially expressed and up regulated proteins were the protein of interest as they expressed in response to biotic stress. These specific proteins were sequenced further to validate the role of differential and up regulated proteins in defense mechanism by MS MALDI-TOF in Sandor Proteomics, Hyderabad.

At 24, 48, and 72h, 16 proteins were expressed in *Pf* treated and control plants challenged with *R. solani*. A 29 kDa protein was up regulated in all the *Pf* treated plants challenged with *R. solani.* The 29 kDa protein expressed at 24h in *Pf* treated plant challenged with *R. solani* was selected for sequencing.

In all the *Pf* treated and BPH challenged plants 3 different proteins of 19, 23 and 30 kDa were expressed. Two protein bands of 19 kDa from 48h and 30 kDa from 96h were selected for MALDI-TOF sequencing.

The sequences were annotated by MASCOT search engine. The following are the identified proteins 1) Fructose-bisphosphatealdolase, 2) Os02g0537700 -*Oryza sativa* (*Japonica* cultivar group), 3) Rubisco Complexed with 2-Carboxyarabinitol-1,5-Bisphosphate.

#### 4.5.1.1. Protein band 1

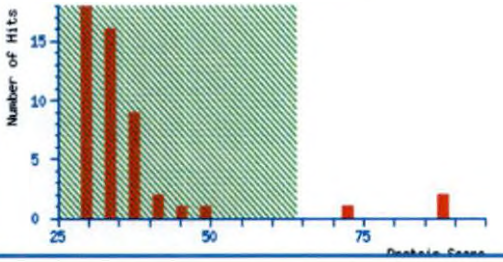
The Protein band 1 selected from the *Pf* primed plant challenged with pathogen was of 29 kDa, showed 84 and 86 per cent homology to 2-cys peroxiredoxin bas, and 2-cys peroxiredoxin bas1, chloroplastic-like respectively. The details of MALDI-TOF search alignment are given in Fig.1 and Table 5.

MATRIX SCIENCE **Mascot Search Results**

```
User            : saroja
Email           : roja.295@gmail.com
Search title    :
Database        : NCBInr 20130310 (23641837 sequences; 8123359852 residues)
Taxonomy        : Oryza sativa (rice) (135471 sequences)
Timestamp       : 11 Mar 2013 at 09:38:57 GMT
Top Score       : 88 for gi|115446541, Os02g0537700 [Oryza sativa Japonica Group]
```

**Mascot Score Histogram**

Protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 64 are significant (p<0.05).



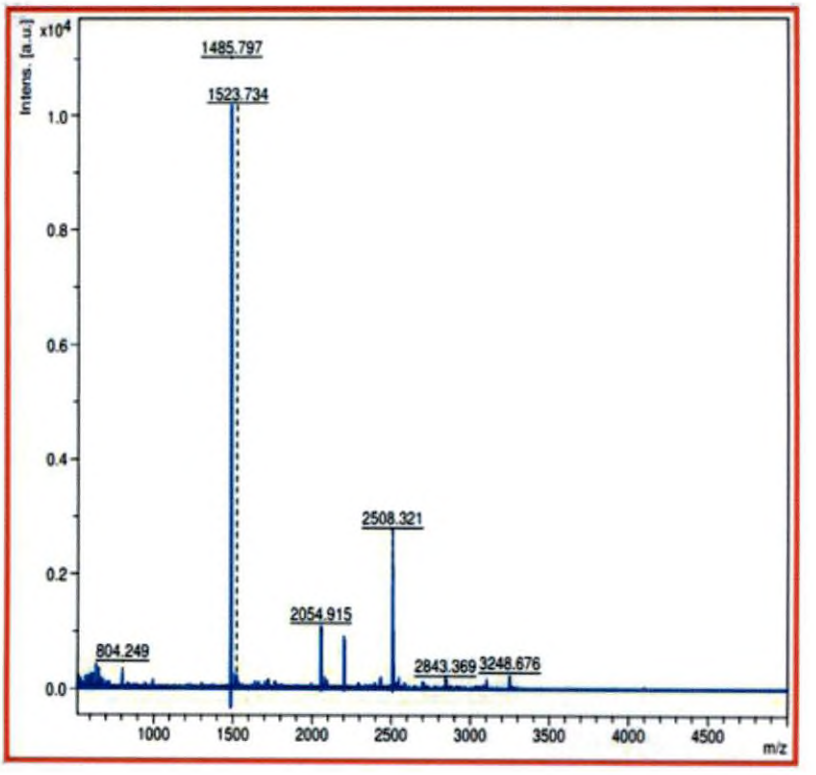




**Fig.1. MALDI-TOF search alignment of protein band 1**

#### 4.5.2.2. Protein band 2

The Protein band 2 selected from the *Pf* primed plant challenged with BPH was of 19 kDa, showed 99, 94 and 81per cent homology to chloroplastic aldolase, fructose-bisphosphate aldolase and peroxidase respectively. The details of MALDI-TOF search alignment were given in Fig.2 and Table 5.

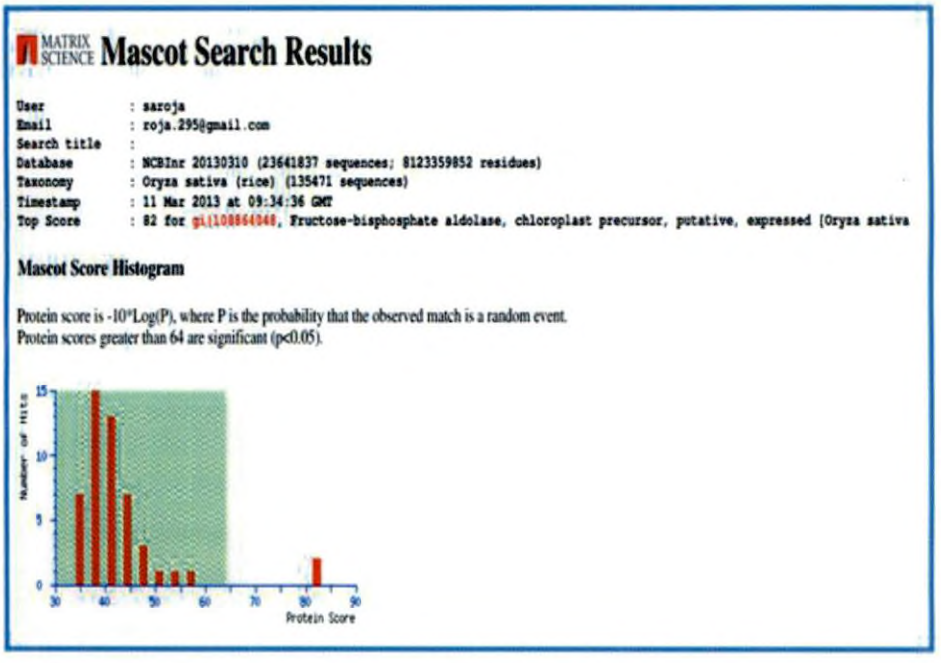
#### 4.5.2.3. Protein band 3

The Protein band 3 selected from the *Pf* primed plant challenged with BPH was of 30 kDa, showed 99 per cent homology to small subunit of ribulose-1, 5-bisphosphate carboxylase and 100 per cent similarity to Os12g0291400 and hypothetical protein OsI_38046. The details of MALDI-TOF search alignment are given in Fig. 3 and Table 5.

### 4.6. Assay of defense related enzyme-Peroxidase

The induced systemic resistance through biochemical and molecular analysis revealed the enhanced activities of defense-related enzyme peroxidase (PO) in the *Pf* treated rice plant against sheath blight disease and BPH insect in rice.

Significant increase in activity of peroxidase upon inoculation with pathogen and insect pest was observed in *Pf* treated plants. The PO activity was increased from 24 to 96h after challenge inoculation in *Pf* primed plants and control plants. The activity was more in *Pf* treated plants compared to control plants at different time intervals. The *Pf* primed plants challenged with pathogen showed 43.62 per cent increase and in BPH challenged showed 21.9 per cent increase at 96h over the control plants (Fig.4)



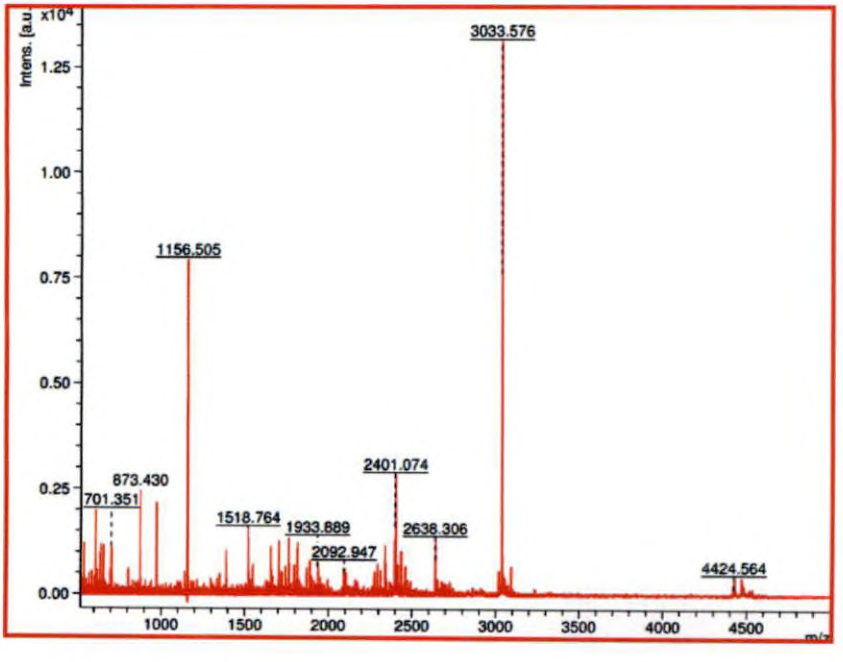
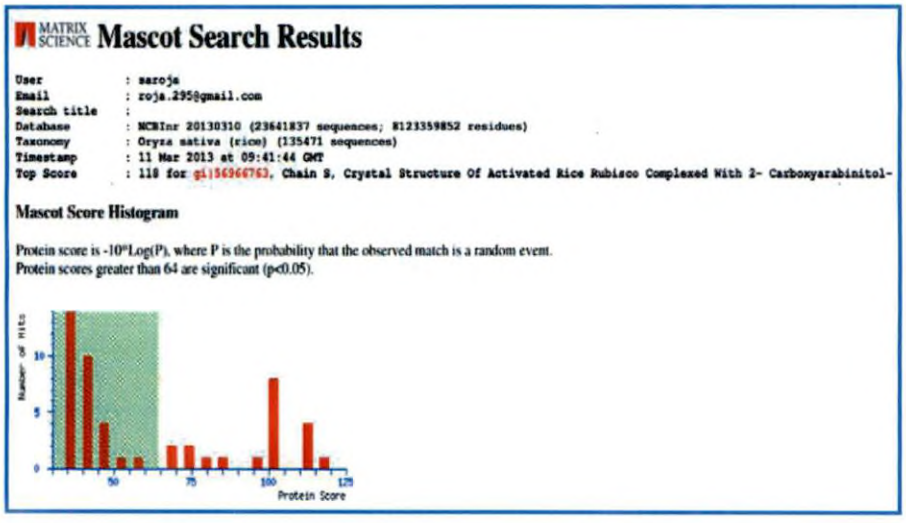




**Fig.2. MALDI-TOF search alignment of protein band 2**



MATRIX SCIENCE **Mascot Search Results**

| | |
|---|---|
| User | : saroja |
| Email | : roja.295@gmail.com |
| Search title | : |
| Database | : NCBInr 20130310 (23641837 sequences; 8123359852 residues) |
| Taxonomy | : Oryza sativa (rice) (135471 sequences) |
| Timestamp | : 11 Mar 2013 at 09:41:44 GMT |
| Top Score | : 118 for gi\|56966763, Chain S, Crystal Structure Of Activated Rice Rubisco Complexed With 2- Carboxyarabinitol- |

**Mascot Score Histogram**

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 64 are significant (p<0.05).



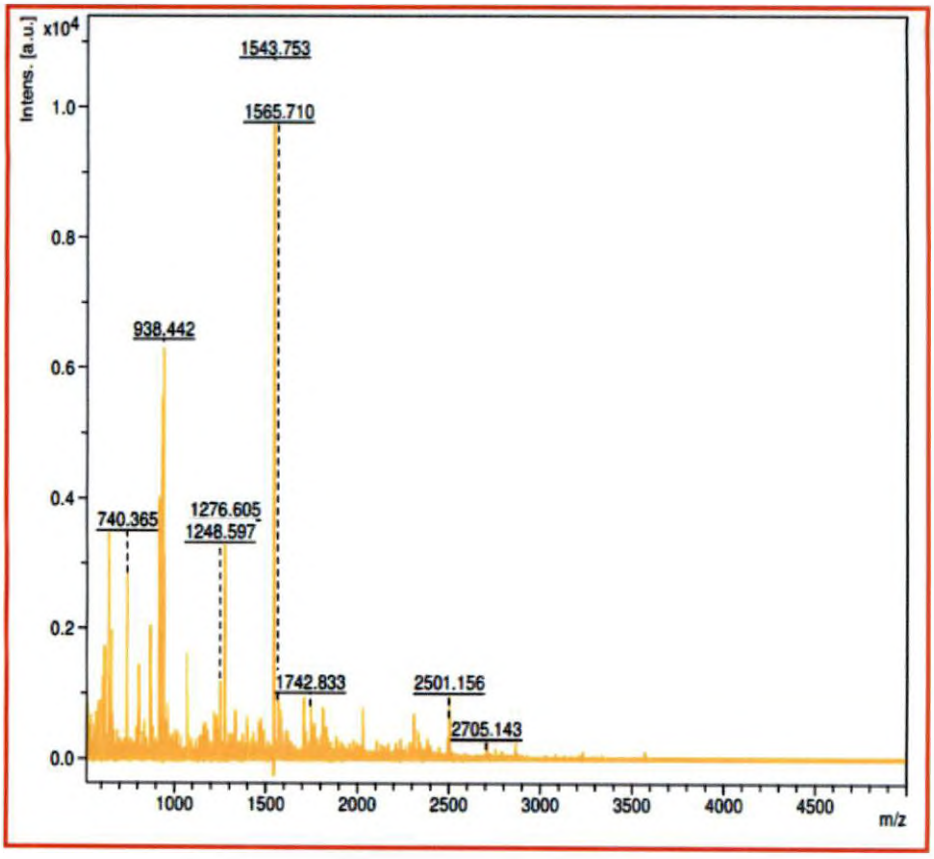



**Fig.3. MALDI-TOF search alignment of protein band 3**

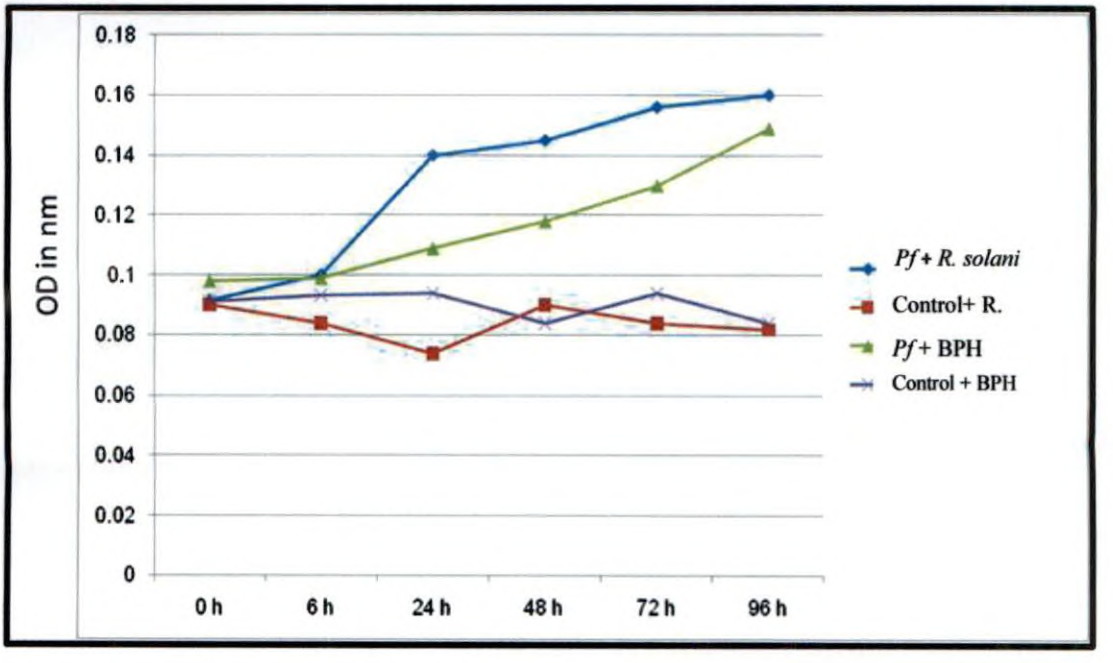



**Fig.4. PO activity in *Pf* treated and control plants challenged with *R. solani* and BPH**

**Table 5. Proteins identified by sequence analysis in response to PGPR priming and biotic stress in rice by MS-MALDI -TOF**

| Spot no. | Name of protein | Identity | Organism | Nature of gene regulation | Accession |
|---|---|---|---|---|---|
| 1(29 kDa) Treatment-*Pf*+*R.solani* | Fructose-bisphosphatealdolase | 100 | *Oryza sativa indica Group* | Differential expression | ABA91631.2 |
| 1 | chloroplasticaldolase | 98 | *Oryza sativa japonica Group* | Differential expression | NP 001065862.1 |
| 1 | fructose-bisphosphatealdolase | 94 | *Zea mays* | Differential expression | ACG36798.1 |
| 1 | peroxidase | 81 | *Oryza sativa indica Group* | Differential expression | ACM17593.1 |
| 2 (19 kDa) Treatment-*Pf*+BPH | Hypothetical protein OsI_07554 | 88 | *Oryza sativa indica Group* | Differential expression | EEC73346.1 |
| 2 | 2-cys peroxiredoxin bas1 | 84 | *Zea mays* | Differential expression | NP 001137046.1 |
| 2 | 2-Cys peroxiredoxin BAS1, chloroplastic | 86 | *Brachypodiumdistachyon* | Differential expression | NP 001148975.1 |
| 2 | 2-Cys peroxiredoxin BAS1, chloroplastic- | 86 | *Zea mays* | Differential expression | ACG 35092.1 |
| 3 ( 30 kDa) Treatment-*Pf*+BPH | RubiscoComplexed 2-Carboxyarabinitol-1 Bisphosphate | 100 | *Oryza sativa japonica Group* | Up regulation | NP 001066606.1 |
| 3 | Os12g0291400 | 100 | *Oryza sativa japonica Group* | Up regulation | NP 001066607.1 |
| 3 | hypothetical protein OsI_38046 | 100 | *Oryza sativa indica Group* | Up regulation | EAY 82834.1 |
| 3 | Os12g0291100 | 100 | *Oryza sativa japonica Group* | Up regulation | NP001066604.1 |
| 3 | small subunit of ribulose-1,5-bisphosph carboxylase | 99 | *Oryza sativa japonica Group* | Up regulation | BAA00539.1 |

### 4.7.2. Functional categorization of identified proteins

The identified proteins were functionally characterized. The proteins were involved in defense mechanism, energy metabolism, and photosynthesis. These identified proteins were previously reported as being involved in the plant response to various environmental or biotic/abiotic stresses. Our result indicates that the expressed proteins have precise role in various pathways which includes both energy metabolism and plant defense. (Fig. 5 and 6)

## 4.8. Protein profiling by Western blot

### 4.8.1. Development of polyclonal antibody

### 4.8.1. Isolation of chitinase from *Trichoderma harzianum*

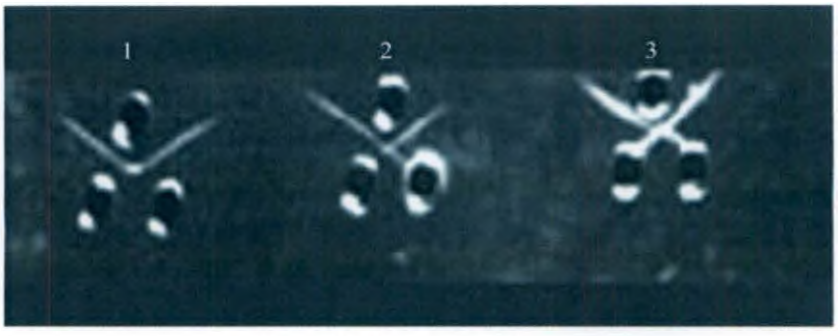
The chitinase enzyme was isolated from *Trichoderma harzianum* and purified by salt solublization and dialysis. The purified chitinase was injected to rabbit to develop polyclonal antibody for chitinase.

### 4.8.2. Development of chitinase in rabbit

The pre immune and post immune serum of rabbit was analyzed to confirm the presence of chitinase antibody by Ouchterlony double-immuno diffusion. There was high titer of chitinase in post immune serum, confirming the presence of chitinase antibody in post immune serum. (Plate 9)

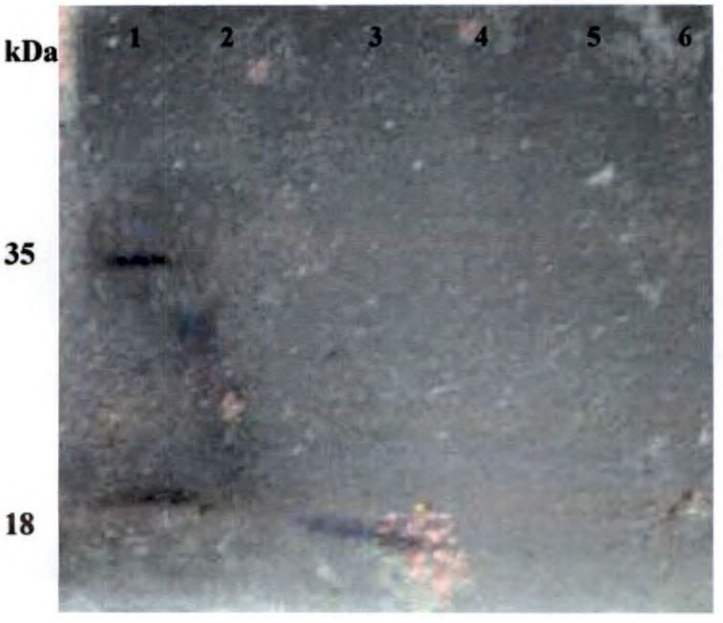
## 4.9. Western Blot Analyses

Western blot analysis of crude protein using rabbit chitinase antisera revealed the presence of chitinase. The 18 and 35 kDa chitinase were expressed in *Pf* primed plants challenged with *R. solani* after 96 hours of inoculation. Seventeen kDa protein band was developed in *Pf* primed plants challenged with *R. solani* and there was no expression in control plants. This confirms the presence of chitinase in PGPR primed plants challenged with *R. solani* (Plate 10).




1: 500 antibody concentration
1: 1000 antibody concentration
1: 3000 antibody concentration

**Plate 9. Detection of antibody titer by Ouchterlony double immuno diffusion**




1: 96 h *Pf* + *R.solani* 2: 96 h control + *R.solani* 3: 72 h *Pf* + *R.solani*
4: 72 h control + *R.solani* 5: 48 h *Pf* + *R.solani* 6: 48 h control + *R.solani*

**Plate 10. Western blot analysis**

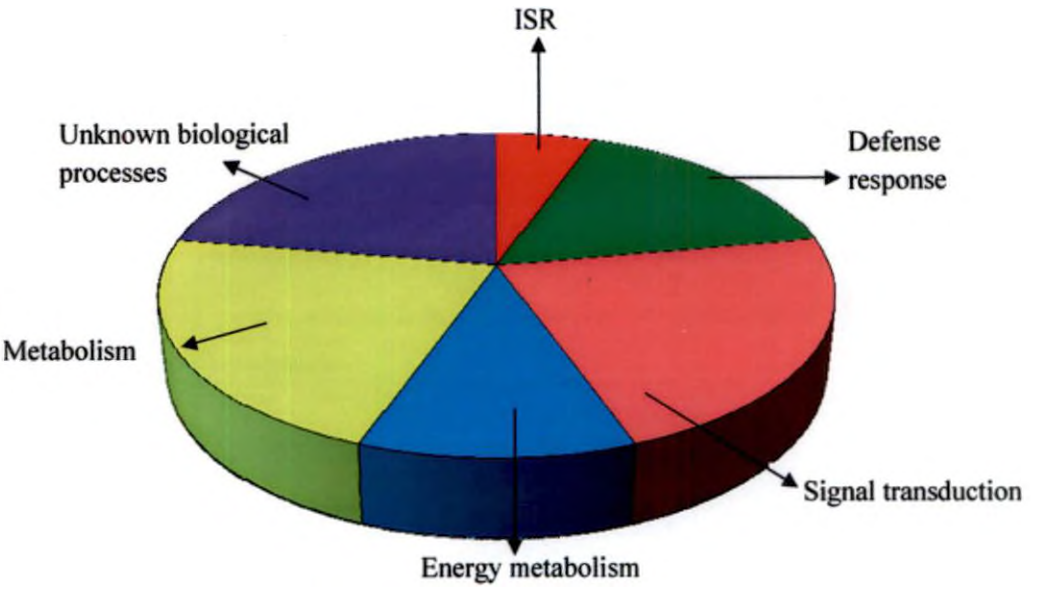



**Fig. 5. Functional categorization of unique sequences expressed in PGPR primed rice plants challenged with *R. solani***

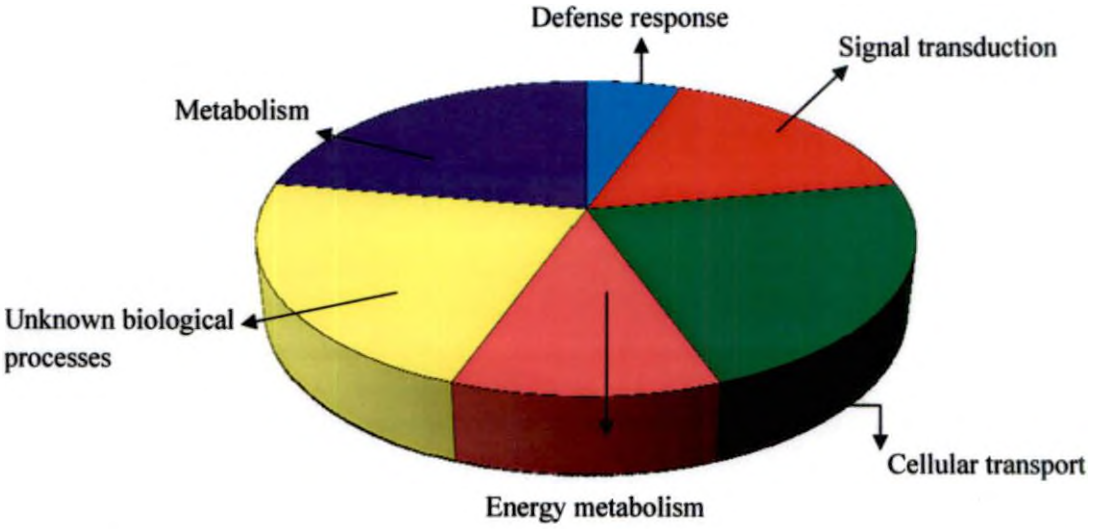



**Fig.6. Functional categorization of unique sequences expressed in PGPR primed rice plants challenged with BPH**


Discussion

# 5. DISCUSSION

Rice is encountered by many biotic and abiotic stresses. Among the various constraints, pests and diseases are major factor for yield loss. The disease sheath blight and insect pest brown planthopper become major setback to rice cultivation throughout the world. In general pest and disease management strategy mainly aims in prevention of outbreak or epidemics through the use of host plant resistance and chemical pesticides. Hazardous effects of chemical pesticides, increasing nature of pathogen and pest challenges classical and molecular breeding approaches.

Research during the last decade indicates the potential option available for plant disease and pest management using biocontrol agents. Recently the focus is on the identification of effective bioagents, especially fluorescent psedumonad bacterial strains which have simple nutritional requirements and excellent root colonizing ability to elicit defense reaction upon infestation by pest and pathogens.

With this background, the current research was undertaken to better understand the molecular mechanisms underlying the Plant growth-promoting rhizobacteria (PGPR) mediated host plant defense.

PGPR colonize the rhizosphere of the many plant species and confer beneficial effect, such as increased plant growth and reduced susceptibility to disease caused by plant pathogenic fungi, bacteria, viruses and nematodes. Some PGPR also elicit physical or chemical changes related to plant defense, a process referred to as 'induced systemic resistance' (ISR). ISR elicited by PGPR has suppressed plant disease caused by a range of pathogen in both greenhouse and field (Kloepper, 2004). The subject of PGPR elicited tolerance to heavy metals also has been reported (Glick, 2003). Biotic stress is a part of biological control and induced resistance (Yang *et al.*, 2009).

## 5.1. Effect of fluorescent pseudomonad strain *Pf* against sheath blight disease

Rice plants when inoculated with *R. solani* produced typical symptoms of sheath blight disease within seven days of inoculation. Disease Intensity was found more on the control plants compared to that of *Pf* treated plants. The per cent disease intensity of *Pf* treated plants was 14.19 per cent and 47.33 per cent in control plants which indicates that

*Pf* has induced resistance in rice plant against *R. Solani* infection through ISR mechanism.

The result obtained can very well be correlated with earlier reports. The plants have endogenous defense mechanisms that can be induced in response to attack by insects and pathogens. It is well known that the defense genes are inducible genes and appropriate stimuli or signals are needed to activate them. Inducing the plant's own defense mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy. The use of fluorescent pseudomonades for controlling soil-borne diseases has been well documented (Paulitz and Loper, 1991; Weller and Cook, 1986).

*P. fluorescens* was found to be effective in chick pea wilt (Vidhayasekaran and Muthamilan, 1999), red rot of sugarcane (Viswanathan and Swamiyappan, 2001) Sheath rot in rice (Saravanakumar, 2006). In the present study, *P. fluorescens* was found to reduce the intensity of sheath blight in rice. *P. fluorescens* could act as strong elicitors of plant defense reactions (Mpiga *et al.*, 1997). Recent studies implies that prior application of *P. fluorescens* strengthen host cell wall structures resulting in restriction of pathogen invasion in plant tissue (Benhamou *et al.*, 2000; Chen *et al.*, 2000). There is a lot of information available on plant-mediated defense reactions induced by fluorescent pseudomonades in plants against pathogen invasion. The present study clearly indicates that the activities of enzymes of phenyl propanoid metabolism and accumulation of PR-proteins in rice have been induced *by P. fluorescens* in response to challenge inoculation with *R. solani.*

The increased accumulation of defense-related proteins in plants treated with *P. fluorescens* before challenge inoculation indicates that seed bacterization triggered the plant to synthesize defense compounds but greater accumulation of defense compounds was observed when *P. fluorescens* treated plants were challenged with the pathogen during the experimental period. In our study PDI was higher in control plant than *Pf* treated plants. The application of *P. fluorescens* in rice has a promising role to induce resistance against sheath blight disease in rice (Ramamoorthy *et al.*, 2002b).

## 5.2. Effect of fluorescent pseudomonad strain *Pf* against BPH

Feeding preference of BPH and the damage of BPH in the rice plants was highly influenced by the application of *Pf*. In our study highest mortality of BPH was observed on plants treated with *Pf*, whereas in control plants there was no mortality. The damage indexing showed more damage on control plants compared to plants treated with *Pf*. Application of *P. fluorescens* significantly reduced the pest incidence in rice. The mechanisms involved in pest resistance accounts for Induced Systemic Resistance (ISR) or Systemic Acquired Resistance (SAR) mechanisms or combination of both in the present study, which correlates with many reports.

Management of insect pest by different pseudomonad strains either as bacterial suspension or through different formulations have been reported by many workers (Kloepper *et al.*, 1980; Zehnder *et al.*, 1997; Vivekannanthan *et al.*, 2004). Pseudomonad strains greatly affect the feeding behaviour of the larvae, and adult. Yaman *et al.* (1999) reported that *P. fluorenscens* suspension spray resulted in 20 per cent weevil mortality in chestnut. *Pseudomonas* treated rice leaves altered the feeding behavior of leaf folder and reduced larval and pupal weight, increased the larval mortality and incidence of malformed adults under *in vitro* conditions (Radjacommare *et al.*, 2002, Saravankumar, 2006).

In our study we found highest mortality of BPH on a plant treated with *P. fluorescens*, whereas as no mortality was found on control plants. Similar result was obtained with damage index also. More damage was observed on control plants as compared to plant treated with *Pf*. The application of *P. fluorescens* significantly reduced the pest incidence in rice. The mechanisms involved in pest resistance may endorse the action of Induced Systemic Resistance (ISR) or Systemic Acquired Resistance (SAR) mechanisms or combination of both in the present study.

## 5.3. Analysis of biometric parameters

The biometric measurements on growth parameters revealed that there is significant increase in shoot length, root length, number of tillers, fresh weight, and dry

weight in *Pf* treated rice plants unlike control plants. There was 4.08 per cent increase in shoot length, 28.01per cent increase in root length, 13.14per cent increase in number of tillers , 14.83per cent increase in fresh weight and 7.06per cent increase in dry weight as compared to control in *Pf* primed plants. Our results also confirmed the role of PGPR in growth promotion as indicated in many reports.

. The results obtained very well correlates with the previous work done by Glick *et al.,* 1999. The mechanisms of growth promotion by fluorescent psedumonads are complex and appears to comprise both changes in the microbial balance in rhizosphere and alteration in the host plant physiology. In our study, it was observed that seed treatment and foliar spray of rice plant with fluorescent pseudomonad strain *Pf* promoted the growth significantly compared to untreated control.

Plant growth promoting rhizobacteria (PGPR) is a group of bacteria that actively colonize plant roots and increase plant growth and yield. The mechanisms by which PGPRs promote plant growth are not fully understood, but are thought to include the ability to produce phytohormones, asymbiotic $N_2$ fixation- against phytopathogenic microorganisms by production of siderophores, the synthesis of antibiotics, enzymes and or fungicidal compounds and also solublisation of mineral phosphates and other nutrients. Significant increase in growth and yield of agronomically important crops in response to inoculation with PGPR have been reported. *Azospririllum*, *pseudomonas* and *Azotobacter* strains could affect seed germination and seedling growth (Kloepper *et al*, 1988). This present investigation confirms the earlier works.

It revealed that under in vitro conditions, seed treatment with PGPR strains improved seed germination, seedling vigor, seedling emergence and seedling strand over the control. These finding may be due to increased synthesis of hormones like gibberellins, which would have triggered the activity of specific enzymes that promoted early germination, such as amylase, which have brought an increase in availability of starch assimilation (Glick *et al.*, 1999). Gamalero *et al.* (2004) found that root length and shoot length was found to increase in all the plants treated with *P. fluorescens* compared to control plants.

Many bacteria have the ability to produce auxins, cytokinins, gibberellins and ethylene (Frankenberger and Arshad, 1995). It has been often been inferred that rhizobacterially produced auxins are responsible for growth promotion. 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 pseudomonad strains. Direct stimulation of plant growth includes the enzyme ACCD secreted by PGPR that can lower plant ethylene level which is produced during biotic and abiotic stress (Glick *et al.*, 1999). In our study, it was observed that fluorescent pseudomonad strain treated plants enhanced the plant height, active tillers, root length, dry weight and wet weight. Thus application of fluorescent pseudomonad strain *Pf* in rice has a promising role in promoting plant growth.

**5.4. Assay of peroxidases**

The induced systemic resistance through biochemical and molecular analysis revealed the enhanced activities of defense related enzyme peroxidase (PO) in the *P.fluorescens* treated rice plant against sheath blight disease and BPH insect in rice.

In our study we found that activity of PO was increased from 6 to 96hours after challenge inoculation in *Pf* primed plants. There was43.62 and 21.9 per cent increase in PO activity over control plants in pathogen inoculated and BPH challenged *Pf* primed plants respectively at 96 hours. Plants inoculated with the pathogen and insect alone had comparatively less PO activity.

Peroxidase is a key enzyme in the biosynthesis of lignin (Bruce and West, 1989). Increased activity of peroxidase has been elicited by fluorescent pseudomonades in plants such as rice ( Reimers *et al.*, 1997; Nandankumar *et al.*, 2001; Radjacommare *et al.*, 2002), black gram( Karthikeyan *et al.*, 2003), groundnut ( Meena *et al.*, 2000), sugarcane (Viswanathan and Samiyappan, 2001) and cucumber (Chen *et al.*, 2000). Peroxidase is capable of oxidizing a variety of phenolics and their presence in insect diets can lead to alkylation of dietary proteins. The oxidation products of corn allele chemicals produced by peroxidases were more toxic than precursors to the maize leafhopper (Dowd and Vega, 1996). In tomato, oxiforms of peroxidases were induced which are specific to particular insect (Stout *et al.*, 1996). Enhanced activity of peroxidase isozymes was

associated with the hypersensitivity response of *Solanum dulcamare* to mite (Bronner *et al.*, 1991).

In general application of fluorescent psedudomonads enhanced the activity of all the enzyme level in rice plants when challenged with pest or pathogen. The enhancement of enzyme was highly special and temporal, as reported by other workers. In bioformulation applied conditions, the plant when challenged with pest or pathogen, the enhancement in enzyme level will be very less. Immediately after challenging with pest or pathogen, the PGPR started to activate all the defense related machineries. We have recorded the enhanced level of PO when compared with control and found that the enhancement was highly need based. The activity of PO was more in pathogen challenged condition when compared to pest attack. Current study proved that PGPR mediation will pave selective temporal and higher expression of PO activity to develop resistance against pest and insect attack.

### 5.5. Identified differential proteins in Rice- PGPR-Pathogen/Pest interactions

#### 5.5.1. Protein band 1

The sequence analysis of protein band1, which was differentially regulated in *Pf* treated plants challenged with *R. solani* was of 29 kDa, showed 84 and 86per cent homology to 2-cys peroxiredoxin bas, and 2-cys peroxiredoxin bas1, chloroplastic-like respectively.

2-Cys peroxiredoxins are a large family of peroxidases which reduce alkyl hydroperoxides and hydrogen peroxide. There discovery in plants (Baler and Dietz, 1996a,b) shows that 2-Cys peroxiredoxins represent an ubiquitously occurring group of enzymes. Basl expression is restricted to green tissues of the shoot (Baler and Dietz, 1996a). The basl gene expression is regulated under the control of the cellular redox state which is in accordance with the anti-oxidant function of the enzyme. The connection between peroxiredoxins and signaling networks has been extensively documented for a number of stress responses, including to pathogen elicitors, insect feeding, wounding, high temperature and ABA associated stomatal closure (Larkindale and Knight, 2002; Apel and Hirt, 2004; Peng *et al.*, 2004; Mateo *et al.*, 2006). The expression of this

peroxiredoxins is more in oxidative stress. The oxidative burst is one of the most rapid defense reactions to pathogen attack in plant (Apel and Hirt 2004). In the present study, the differential expression of protein band 1was noticed in the PGPR treated rice leaf sheath tissues which were challenged with pathogen when compared to control. From this study it is clear that 2-Cys peroxiredoxins was expressed to overcome the pathogen attack as a defense mechanism.

### 5.4.1. Protein band 2

The sequence analysis of protein band 2 which is also differentially expressed in *Pf* treated plants challenged with BPH and was 19 kDa, showed 99, 94 and 81 per cent homology to chloroplastic aldolase, fructose-bisphosphate aldolase and peroxidase respectively.

The chloroplastic aldolase and fructose-bisphosphate aldolase belong to class I adolases. Plant aldolases are class-I type enzymes that possess a key lysine residue in the catalytic site required for the formation of a covalent intermediate in the form of a Schiff base with its substrate prior to the aldol cleavage (Anderson and Advani, 1970). It plays a vital role in photosynthesis mostly in Calvin cycle. In the present study, the differential expression of protein band 2 was noticed in the PGPR treated rice leaf sheath tissues when compared to control. It is clear that, photosynthetic activity was higher in order to attain greater growth and possible link with plant defense.

Peroxidases are frequently associated with plant defense against pathogens. They are responsible for the radical dehydrogenation of sinapil alcohol and koniferyl alcohol during the lignin synthesis. They are represented by many of the isoenzymes. Peroxidase polymorphism could be also used as a biochemical marker related to the different levels of field resistance (Lebeda, and Reinink, 1994). Peroxidase participates in processes which occur in the extracellular matrix (Buonario and Montalbini, 1993). Their association with the cell wall was confirmed. Peroxidases remove the toxic hydrogen peroxide from tissues; participate in synthesis of phenolic compounds and in the building of intermolecular bonds during the organization of the cell wall at the sites of infection by pathogens (Repka and Slovakova 1994). The production of phenolic compounds include synthesis of chinons, tanins, melanins. Tanins and melanins are dihydroxyphenol and

chinon oligomers, these are toxic for pathogens. Lignin and suberin are involved in structural defenses. Peroxidase also participates in the synthesis of ethylene, the concentration of which increases frequently in pathogenesis process (Tudzynski 1997). Generally peroxidases enhance their activity after a pathogen attack, because they participate in defensive lignification and synthesis of phenolic compounds effective against pathogens (Nicholson and Hammerschmidt 1992). In our study, earlier and increased peroxidase activity has been recorded in *P. fluorescens* isolate *Pf*-treated plants challenged with the BPH. Chen *et al.* (2000) reported the higher PO activity in cucumber roots treated with *P. Corrugata.* The present study also indicates that a PO was prominently expressed in *P.fluorescens* treated plants against *R. solani.* This unique PO induced by *P. fluorescens* isolate *Pf* might have contributed to induced defense in rice sheath tissue against infection by BPH.

### 5.4.3. Protein band 3

The sequence analysis of protein band 3 which was differentially expressed in *Pf* treated plants challenged with BPH was of 30kDa, showed homology to small subunit of ribulose-1, 5-bisphosphate carboxylase with 99per cent identity. It showed 100per cent similarity to both Os12g0291400 and hypothetical protein OsI_38046, which are hypothetical proteins and its role in defense mechanism is not yet known.

The level of RuBisCO proteins, one of the important enzyme in photosynthesis, is known to be reduced in infected plants cell and during pest attack due to chloroplasts degaration (Agrios, 1997). RuBisCO proteins were also reported to be increased in rice leaf sheath by wounding stress (Shen *et al.*, 2003). In the present study, the differential expression of protein band 3was noticed in the PGPR treated rice leaf sheath tissues when compared to control. It is clear that, this is strongly due to increased photosynthetic activity to attain greater growth and possible link with plant defense.

## 5.6. Analysis of protein by Western blot

### 5.6.1. Isolation of chitinase from *Trichoderma harzianum*

The chitinase enzyme was isolated from *T.harzianum* and was purified by salt separation and dialysis. The purified chitinase, which is the antigen was injected to rabbit

to develop polyclonal antibody for chitinase. The presence of antibody for chitinase was detected post immune serum collected from rabbit by Ouchterlony double immuno diffusion. Good titer was detected in Ouchterlony double immuno diffusion. The antibody developed was used for western blot analysis.

### 5.6.2. Western Blot Analyses

The results of the western blot analysis also revealed that about 18 and 35 kDa proteins were developed in *Pf* primed plants challenged with *R. solani* after 96hour of inoculation and 17 kDa protein band was developed in *Pf* primed plants challenged with *R. solani* after 72h. This confirms the presence of chitinase which are group of pathogenesis-related proteins expressed in PGPR primed plants challenged with *R. solani.* This indicated that chitinase was expressed to develop resistance in plant against pathogen.

Most of Chitinase were having molecular mass in the range of 14 kDa and 43 kDa. Chitinase could be isolated from *Cicer arietinum* (chickpea) (Saikia*et al.*, 2005), *Cucumis sativus* (cucumber), *Hordeum vulgare* (barley) (Kirubakaran and Sakthivel, 2006), *Nicotiana tabacum* (tobacco) (Pu *et al.*,1996), *Phaseolus vulgaris* (black turtle bean) (Chu and Ng, 2005), *Solanum lycopersicum* (tomato) (Wu and Bradford, 2003) and *Vitis vinifera* (grapes) (Sluyter *et al.*, 2005). It is also reported that Chitinase gene are also expressed in response to stress like cold up to -2 to -5°C (Yeh *et al* ., 2000). These Chitinases have significant antifungal activities against plant pathogenic fungi like *Alternaria* sp. for grain discoloration of rice, *Bipolaris oryzae* for brown spot of rice, *Botrytis cinerea* for blight of Tobacco, *Curvul arialunata* for leaf spot of clover, *Fusarium oxysporum*, *F. udum*, *Mycosphaerella arachidicola* , *Pestalotia theae* for leaf spot of tea and *Rhizoctonia solani* for sheath blight of rice (Chu and Ng 2005; Saikia *et al* ., 2005; Kirubakaran and Sakthivel, 2006). The mode of action of PR-3 proteins is relatively simple *i.e.* Chitinases cleaves the cell wall chitin polymers *in situ*, resulting in a weakened cell wall and rendering fungal cells osmotically sensitive (Jach *et al.*, 1995).

Even though overexpression of PR-proteins may not result in complete resistance to a specific pathogen, the degree of protection afforded by specific combinations may be sufficient to be useful agronomically.

# *Summary*

# 6. SUMMARY

Biotic stress is one of the inherent constraints that affect agricultural productivity worldwide. It is estimated that biotic stress potentially reduce crop yield around the world. Rhizobacterial strains are found to increase plant growth after inoculation in seed and therefore called Plant Growth Promoting Rhizobacteria (PGPR). Plant Growth Promoting Rhizobacteria including fluorenscent Pseudomonads is capable of surviving and colonizing the rhizosphere of all field crops. They promote plant growth by producing gibberellins and cytokinins.

To assess the Induced Systemic Resistance in rice the plants were challenged with sheath blight pathogen *R. solani* and BPH insect forty-five days after sowing during active tillering stage.

Rice sheath blight pathogen *Rhizoctonia solani* was isolated from infected leaf sheath. The per cent disease index (PDI) was checked for the plants challenged with *R. solani* after seven days of inoculation. PDI of control plant was found more as compared with *Pf* treated plant. Thus application of fluorescent psedumonad strain *Pf* in rice has a promising role to induce resistance against sheath blight disease in rice.

For assessing the efficiency of pseudomonas treatments on rice in pest attack, BPH, were released. Feeding preference of BPH and the damage of BPH on rice plants was highly influenced by the application of *Pf.* Nearly 28 per cent mortality was observed in *Pf* treated plants. There was no mortality in control plants. The damage caused due to BPH was rated by using 0-9 scale of Standard Evaluation System for rice, IRRI (2002). Observations were taken seven days after release of insect. There was no damage on plants treated with *Pf* where as control plants were fed by BPH. The *Pf* treated rice plants showed high level of resistance to BPH attack. There was no damage on plants treated with *Pf* but control plants were susceptible.

Biometric parameters were measured in control and *Pf* treated plants. There is significant difference in shoot length, root length, No. of tillers, fresh weight, and dry

weight. PGPR help to attain high vigour in plant. There was 4.08 per cent increase in shoot length, 28.01 per cent increase in root length, 13.14 per cent increase in number of tillers, 14.83 per cent increase in fresh weight and 7.06 per cent increase in dry weight in *Pf* primed plants compared to control plants. Our results revealed the role of PGPR in growth promotion.

Analysis of protein was carried out by SDS-PAGE and Western blot. The proteins were separated based on molecular weight by SDS-PAGE in different samples collected at different time intervals. SDS-PAGE analysis of proteins from leaf extract of rice showed clear differences among *Pf* treated and control plants challenged with *R. solani.* At 6h after inoculation, 16 proteins were expressed in *Pf* treated plants and 12 in control plants. At 24, 48, and72h, proteins were up regulated in *Pf* treated plants unlike control plants. The banding pattern of proteins expressed at 96h after inoculation was same in both *Pf* treated and control plants.

SDS-PAGE analysis of proteins from leaf extract of rice showed clear differences among *Pf* treated and control plants challenged with BPH. At 6, 24, 48, 72 and 96h after challenge inoculation, all the proteins were up regulated in *Pf* treated plants over the control plants. There was expression of 25 proteins in all the *Pf* treated plants where as only 13 in control plants. There is up regulation and differential expression in *Pf* treated and control plants in all the treatments. The 19, 23 and 30 kDa proteins were differentially regulated in all *Pf* treated plants, as these proteins were specifically expressed in *Pf* treated plants and not in control plants.

The 29 kDa protein expressed at 24h in *Pf* treated plant challenged with *R. solani* was selected for sequencing. Two protein bands 19 kDa from 48h and 30 kDa at 96h were selected for MALDI-TOF sequencing. The sequences were annotated by MASCOT search engine. The following proteins were identified, Fructose-bisphosphate aldolase, Os02g0537700 -*Oryza sativa* (*Japonica* cultivar- Group) and Rubisco Complexed With 2- Carboxyarabinitol-1,5-Bisphosphate.

The Protein band 1 selected from the *Pf* primed plant challenged with pathogen was of 29 kDa, showed 84 and 86 per cent homology to 2-cys

peroxiredoxin bas, and 2-cys peroxiredoxin bas1, chloroplastic-like respectively. The sequence shows 84 per cent homology with 2-cys peroxiredoxin bas1. These proteins play vital function in defense reactions to pathogen attack in plant.

The Protein band 2 selected from the *Pf* primed plant challenged with BPH was of 19 kDa, showed 99, 94 and 81per cent homology to chloroplastic aldolase, fructose-bisphosphate aldolase and peroxidase respectively.

The Protein band 3 selected from the *Pf* primed plant challenged with BPH was of 30 kDa, showed 99 per cent homology to small subunit of ribulose-1, 5-bisphosphate carboxylase and 100 per cent similarity to Os12g0291400 and hypothetical protein OsI_38046 proteins and their role in defense mechanism is unknown.

Western blot analysis of protein using rabbit chitinase antisera revealed the presence of chitinase. The 18 and 35 kDa chtinase were expressed in *Pf* primed plants challenged with *R. solani* after 96 hours of inoculation. Seventeen kDa protein band was developed in *Pf* primed plants challenged with *R. solani* and there was no expression in control. This confirmed the presence of chitinase in PGPR primed plants challenged with *R. solani,* indicated chitinase, a pathogenesis –related protein was induced to develop resistance in plant against pathogen.

Significant increase in activity of peroxidase upon inoculation with pathogen and insect pest was also observed in *Pf* treated plants. The PO activity was increased from 24 to 96h after challenge inoculation in *Pf* primed plants over control plants. The activity was more in *Pf* treated plants compared to control plant at different time intervals. The *Pf* primed plants challenged with pathogen showed 43.62 per cent increase and in BPH challenged *Pf* treated plants showed 21.9 per cent increase at 96h over the control plants.

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# Annexures

## ANNEXURE I

### Reagents required for protein extraction

**1. 0.1 M Sodium citrate buffer (100 ml) pH 5.0**

Sodium citrate : 2.94 g

**0.1N citric acid (20ml)**

Citric acid : 0.42 g

Dissolve the sodium citrate in 50 ml of distilled water and make up the volume 100 ml .With the help of citric acid maintain the pH of the solution up to 5.0.

## ANNEXURE II

**Reagents required for SDS-PAGE**

**1. 0.1 N NaOH (250 ml)**

NaOH : 1.0 g

Dissolve in distilled water and make up the volume 250 ml

**2. Acrylamide- Bis acrylamid stock (100 ml)**

Acrylamide : 29.2 g

N'N' Bis methyline acrylamide : 0.8 g

Dissolve in 80ml of distilled water make up to 100 ml, filter and store at $4^0$c in dark.

**3. Seperating gel buffer (pH-8.8)**

1.875 M Tris HCL : 22.7 g

Dissolve in 80ml of distilled water, adjust the pH 8.8 with 1N HCL,make up to 100 ml with distill water, store at $4^0$c.

**4. Stacking gel buffer (pH6.8) (100 ml)**

0.6 M Tris HCL : 9.45 g

Dissolve in 80ml of distilled water, adjust the pH 6.8 with 1N HCL, make up to 100 ml with distill water, store at $4^0$c.

**5. 10% SDS 10ml**

SDS : 1 g

Dissolve 1 g SDS in distilled water with gentle stirring, make to 10 ml with distilled water. Store at room temperature.

**6.0.1% APS 1 ml**

APS : 100 mg

Dissolve in 1 ml of distilled water. Prepare freshly at each time.

**7. Tank buffer 1l**

| | | |
|---|---|---|
| Glycine | : | 14.4 g |
| Tris base | : | 3.09g |
| Distill water | : | 1l |

Use 2-3 times store at room temperature.

**8. 5X Sample buffer (pH-6.8) 10 ml**

| | | |
|---|---|---|
| 1.5M Tris HCL | : | 2ml |
| Glycerol | : | 5ml |
| β mercaptoethanol | : | 2.5ml |
| SDS | : | 1 g |
| 1% bromophenol blue | : | 0.5 ml |

**9. Protein staining solution 100ml**

| | | |
|---|---|---|
| Coomasssie brilliant blue R-250 | : | 0.1 g |
| Methanol | : | 40ml |
| Acetic acid | : | 10ml |
| Water | : | 50ml |

First dissolve dye in methanol and proceed.

**10. Destaining solution 100ml**

| | | |
|---|---|---|
| Methanol | : | 40ml |
| Acetic acid | : | 10ml |
| Water | : | 50ml |

| Components | 12% Separating gel (25ml) | 4% Stacking gel (10ml) |
|---|---|---|
| Distilled water | 8.3 ml | 6.8 ml |
| Acrylamid and Bisacrylamid mixture | 10 ml | 1.7 ml |
| Tris buffer | 6.3 ml | 1.25 ml |
| 10% SDS | 250µl | 100 µl |
| 0.1% APS | 250 µl | 100 µl |
| TEMED | 10 µl | 10 µl |

# PROTEOME ANALYSIS OF INDUCED SYSTEMIC RESISTANCE MEDIATED BY PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) IN RICE FOR BIOTIC STRESS

**By**

**Shinde Subhashini Ganesh**
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## ABSTRACT OF THE THESIS

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**CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY**
**COLLEGE OF HORTICULTURE**
**VELLANIKKARA, THRISSUR - 680 656**
**KERALA, INDIA**
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## ABSTRACT

Rice (*Oryza sativa* L.), is vulnerable to a number of pest and diseases. Among them sheath blight disease caused by *Rhizoctonia solani*, and insect pest, brown planthopper (BPH) (*Nilapavata lugens*) are the most devastating agents and major challenge to rice cultivation. Approximately 30 per cent yield loss has been reported due to above said disease and pest. In this context, the present study entitled 'proteome analysis of induced systemic resistance mediated by plant growth promoting rhizobacteria (PGPR) in rice for biotic stress' was carry out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2011-2013, with the objective to identify and characterize the interacting proteome in inducing systemic resistance in rice mediated by PGPR during pathogen and insect attack.

Plant growth-promoting rhizobacteria (PGPR) are associated with plant roots and augment plant productivity and immunity. However, recent work by several groups shows that PGPR elicit physical or chemical changes related to plant defense, a process referred as 'induced systemic resistance' (ISR). ISR induced by PGPR has suppressed plant diseases caused by a range of pathogens in both greenhouse and field (Yang *et al.*, 2009). Jyothi (PTB -39), a popular rice variety of Kerala and susceptible to blight and BPH was used as the experimental material. The pot culture experiment was carried out by direct sowing. The biometric observations were taken on control plants and the plants treated with *Pseudomonas fluorescens Pf1* (KAU culture) 30 days after sowing. Significant increase was observed in shoot length, root length, number of tillers, fresh weight and dry weight in *Pf* primed rice plants. Both *R. solani* and BPH were inoculated forty five days after sowing, to check the efficacy of *Pf* strain against the pathogen and insect.

Protein analysis was carried out to study the molecular mechanisms operating behind the PGPR mediated pest and disease resistance and growth promotion. Total Proteins were isolated from rice plants treated with and without *Pf*

and challenged with pest and pathogen at 0, 6, 24, 48, 72, and 96 hours after inoculation (HAI). The protein samples were analyzed by SDS-PAGE gel system. The comparison analysis of relative abundances of protein bands between inoculated and non inoculated samples were carried out. At 24, 48, and 72h, 16 proteins were expressed in *Pf* treated and control plants challenged with *R. solani*. A twenty nine kDa protein was up regulated in all the *Pf* treated plants challenged with *R. solani* and this was selected for sequencing. In all the *Pf* treated and BPH challenged plants 3 different proteins of 19, 23 and 30 kDa were expressed. Two protein bands of 19 kDa from 48h and 30 kDa from 96h were selected for MALDI-TOF sequencing.

These three proteins bands were sequenced by MS MALDI-TOF in Sandor Proteomics, Hyderabad. Protein band 1 of 29 kDa, showed, 98 per cent homology to chloroplastic aldolase, fructose-bisphosphate aldolase and 81 per cent homology to peroxidase. The protein band 2 of 19 kDa, showed 84 and 86 per cent homology to 2-cys peroxiredoxin bas and 2-cys peroxiredoxin bas1, respectively. The Protein band 3 of 30 kDa, showed 99 per cent homology to small subunit of ribulose-1, 5-bisphosphate carboxylase and 100 per cent similarity to hypothetical proteins Os12g0291400 and OsI_38046.

Western blot analysis was also carried out to further confirm the presence of PR protein chitinase. The analysis confirmed the presence of chitinase of molecular weight 17 kDa, 18 kDa, and 35 kDa in PGPR primed plant challenged with *R.solani*. The PO assay was also carried out to check the activity of peroxidase enzyme in both control and PGPR primed plants challenged with BPH and pathogen. PO activity was increased from 6 to 96 h after challenge inoculation in *Pf* primed plants. There was 43.62 and 21.9 per cent increase in PO activity over control plants in pathogen inoculated and BPH challenged *Pf* primed plants at 96hours. The result obtained gave information for further elucidation of candidate genes operating in signal transduction pathways mediated by PGPR during ISR to biotic stress.