ANTIPHYTOPATHOGENICITY OF BIOAGENTS AND EXPRESSION ANALYSIS OF SELECTED DEFENCE GENES IN RICE IN PRESENCE OF ACTINOBACTERIA AND *Rhizoctonia solani*

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INTRODUCTION

Plants are susceptible, at all stages of growth and development, to attack by various types of disease causing microorganisms. Thus, a major focus within the discipline of plant biology is the interaction between plants and pathogens (Walton, 1997). Plant constitutes an excellent ecosystem for interaction with microorganisms. The most interesting part is that plant interacts with both beneficial and harmful (pathogenic) microorganism.

Plants, like human and other animals also get infected, but they have sophisticated defence response against microbes. The response is mediated by several messenger molecules that are activated by genes (Montesino *et al*., 2002). Beneficial bacteria act as biological control agents and also promote plant growth (Lai *et al*., 2001). Several implications for the management of plant diseases are derived from knowledge on the specificity of plant-microbes interactions (Baker, 1997). So study of plant diseases caused by pathogens (infectious diseases) and environmental conditions (physiological factors) is important because plant diseases affect humans and animals health also.

Biotic stress such as pathogen attack severely impairs plant growth and development and limits crop production. Among the list of different plants and their diseases, rice (*Oryza sativa* L.) is the most widely cultivated food crop in the world. Rice is one of the three major cereals in the world. Rice cultivation is often subjected to several biotic stresses of which diseases like blast, sheath blight (ShB), stem rot, and bacterial blight are important. ShB of rice is an important soil-borne fungal disease (*Rhizoctonia solani* Kuhn) causing up to 40 per cent of yield losses annually, especially when susceptible varieties are cultivated.

Rhizoctonia solani Kuhn was originally reported causing sheath blight of rice in Japan (Miyake, 1910). *R. solani* is a soil borne fungal pathogen which cause damage and spread disease, which ultimately affect the rice production. In order to survive and adapt to this stress, plants must modulate various physiological and metabolic responses based on the stress signals (Bohnert *et al.,* 1999). Thus identifying and understanding these mechanisms is crucial for the development of tolerant commercial cultivars. Resistance to biotic stress involves a complex of mechanisms working in combination to avoid or tolerate pathogen attack. Many genes are reported to be involved in stress conditions, which function not only in directly protecting the cells but also in the regulation of gene expression and signal transduction. Molecular basis for plant resistance to biotic stress remains unclear, because of the reason that several regulatory mechanisms are involved in the stress signal pathways thus making biotic stress a multigenic character. Therefore, for engineering resistance to biotic stress in plants, it is important to understand this complex networks for which it becomes necessary to identify and characterize the genes that respond to biotic stress.

A biological agent or bio-agent is a bacterium, virus, or fungus that can be used as a biological weapon in the biological warfare. In addition to these natural antagonists, biological toxins are also included among the bio-agents. More than 1,200 different kinds of potentially effective bio-agents have been described and studied to date.

Biocontrol agents have the ability to control numerous foliar, root, and fruit pathogens and even invertebrates such as nematodes. However, this is only a subset of their abilities. We now know that they also have the ability to ameliorate a wide range of abiotic stresses, and some of them can also alleviate physiological stresses such as seed aging. They can also enhance nutrient uptake in plants and can substantially increase nitrogen use efficiency in crops. These plant growth promoting abilities may be more important to agriculture besides disease control. Some strains also have abilities to improve photosynthetic efficiency and probably respiratory activities of plants. All of these capabilities are a consequence of their abilities to reprogram plant gene expression, probably through activation of a limited number of general plant pathways (Shoresh *et al*., 2010).

New biotechnological products are currently being developed based on stimulation of the plant defence response, and on the use of plant beneficial bacteria for biological control of plant diseases and for plant growth promotion (Altman, 1998). One of the powerful techniques of molecular biology to identify these expression levels of genes regulating the three way interaction involving the host, pathogen and the bioagent is quantitative real time-polymerase chain reaction (qRT-PCR). A comprehensive analysis of induced and repressed genes during the three way interaction will lead to a better understanding of the molecular processes involved in infection and resistance, and will contribute to the knowledge-base of dynamic plant resistance strategies.

Genome-wide analysis of mRNA abundance showed that expression of 5-30 per cent of the genes assayed was modulated by stress condition (Rabbani *et al*., 2003; Wang *et al*., 2003). In some cases, differential regulation of specific genes and pathways has been associated with improved adaptation of crop genotypes to different stress (Zhang *et al*., 2004). Advancement in technologies such as DNA sequencing methodologies, throughput platform DNA array, northern blotting, subtractive hybridization, real-time PCR *etc.* have tremendously contributed to our knowledge of transcriptomes. The advent of real-time PCR technology has significantly changed the field of quantifying gene expression in plant molecular biology research.

Few bioagents have the potential to induce systemic resistance in a host plant by activating defence pathways. Seed priming with rhizobacterial strains resulted in control of disease as well as facilitates plant growth promotion. Thus, beneficial microbes play dual role as biocontrol agents and release metabolites for plant growth promotion. During this interaction several defence genes are differntially expressed or repressed.

With these views in the background, the following objectives were planned in the present investigation.

1. Identification of potential biocontrol isolates of *Trichoderma*, *Rhizobacteria* and Actinomycets against the rice sheath blight pathogen, *Rhizoctonia solani.*
2. Study on expression of defence genes/ PR genes in the host during its interaction with bioagent and the pathogen.

REVIEW OF LITERATURE

Rice (*Oryza sativa* L.) is one of the world's most important crops, providing staple food for nearly half of the global population. It is cultivated in at least 114, mostly developing countries and is the primary source of income and employment for more than 657.5 million households in Asia and Africa (FAO, 2012). The world population is six billion and will count eight billion by the year 2020. Of the 840 million people suffering from chronic hunger, over 50 per cent live in areas dependent on rice production.

Almost 90 per cent of the rice is grown and consumed in Asia (Brar and Khush, 2002). About 80 per cent of the world's rice is produced on small farms, primarily to meet family needs, and poor rural farmers account for 80 per cent of all rice producers (FAO, 2012). The ever increasing human population especially in the developing countries along with various abiotic and biotic stresses has posed a challenge to boost the rice production in limited cultivable land (Beck and Ulrich, 1993).

The majority of the rice (90%) is being produced in Asian countries with China and India being the major producer. The other major rice producing countries are Indonesia, Bangladesh, Vietnam, Thailand, Myanmar, Philippines, Brazil, and Japan (IRRI, 2008).

Rice production in India is an important part of the rural/agrarian economy. India is the world's second largest producer of white rice, accounting for 20 per cent of all world rice production. Rice is India's preeminent crop and is the staple food of two thirds of population of the country with an annual production of 110 mt. To meet the demand of ever increasing population, the annual production of rice needs to be enhanced to 125 mt by 2020. India is the world's second largest producers of rice, followed by China.

The following account summarizes present knowledge on the diseases, pathogens, their interactions, the effect of bioagents and their mechanisms in disease suppression as well as plant growth promotion. Amongst the rhizopshere microorganisms, the plant growth promoting rhizobacteria (PGPR) as well as other biological agents, like trichoderma and actinomycetes has been considered important in sustainable agriculture due to their plant growth promotional ability as well as biocontrol potential. (Carla *et al*., 2008; Reddy *et al*., 2010)

Biocontrol agent has emerged as the consortium of beneficial soil microorganisms involved in the control of a number of plant diseases and pests by virtue of their ability to synthesize a wide range of antagonistic secondary metabolites. These ubiquitous microorganisms can be an integral component of management practices to achieve sustainable yields.

Literature pertaining to the plant growth promotion, biocontrol activity, mechanisms of actions of bioagents is reviewed herein. Also it included the molecular interaction of bioagent with plant against particular phytopathogen, which gives the clue for the type of resistance pathway involved and genes involved in defence mechanism.

2.1 Disease symptoms and the pathogen

Rice cultivation is often subjected to several biotic stresses of which diseases like blast, sheath blight (ShB), stem rot, and bacterial blight are important. ShB of rice is an important soil-borne fungal disease (*Rhizoctonia solani* Kuhn) causing up to 40 per cent of yield losses annually, especially when susceptible cultivars are grown (Prasad and Eizenga, 2008).

Sheath blight or banded blight/ sclerotial disease is an economically important disease and is reported from many countries including India. Initially the disease was identified as minor, but is now positioned next to blast in the terms of economic importance (Manibhushanrao, 1995).

The disease manifests initially as water soaked lesions on sheaths of lower leaves near water line. The dense crop canopy and high relative humidity (>95%) in the canopy usually favors the ShB development. As the disease advances; the lesions expand and are expressed as a bleached appearance with a brown border. Under ambient conditions, the disease assumes severe form and chaffiness of lower grains in the panicle is usually seen. The pathogen survives in the form of sclerotial bodies in the soil for several years, on stubbles of the previous season's crop and on weeds (Kozaka 1961; Kobayashi *et al.* 1997).

The rice ShB pathogen produces several cell wall degrading enzymes. Immersion of rice sheaths in these enzymes resulted in breaking of callus, sheath cell, organelle, and also in cell wall cracking and mitochondrial damage (Zhang *et al*, 2005).

R. solani Kuhn was originally reported causing sheath blight of rice in Japan at the beginning of this century (Miyake, 1990). However, rice sheath blight has emerged into a major disease only during the past two decades. Nowadays, the disease is second only to rice blast (*Pyricularia oryzae*) in terms of economic importance in major rice growing countries.

The emergence of *R. solani* as a major rice pathogen has been attributed to the intensification of rice cropping system characterized by the development of new, short statured, high tillering, high yielding varieties and an increase in nitrogen fertilization inducing a favorable micro-climate for pathogen. The ShB pathogen can infect the rice crop at any stage of growth from seedling to flowering by different inoculum sources. Among the different types of symptoms ShB is the most prominent and common one. Other symptoms on rice include pre and post emergence seedling blight, banded leaf blight, panicle infection, and spotted seed (Acharya *et al*., 1997).

Pot culture studies on the susceptibility of rice seedlings to *R. solani* revealed that disease incidence and development was rampant on 20 to 30 days old rice seedlings compared to seedlings of 30 to 40 days old under artificially inoculated conditions (Sharma and Thrimurty, 2006).

2.2 Pathogenicity of *Rhizoctonia solani*

Rice ShB symptom production under artificial conditions depends on the method of inoculation. Of different inoculation techniques such as single grain insertion, single sclerotium insertion, and mycelial suspension injection, single sclerotium insertion method was the most effective with highest ShB symptoms (68.5 to 80.0%), lesion length (2.45 to 4.75 cm) and percent disease index (32.5-43.5) followed by single grain insertion technique (Chakraborty *et al*., 2006).

Maximum disease severity was observed when sheaths and leaves were inoculated with 7-day-old propagules of the pathogen (Sharma and Thrimurty, 2004).

The amount of *R. solani* inoculum plays a major role in uniform ShB disease development. Inoculum at the rate of 0.2 mg when placed inside the leaf sheath with a few drops of sterile water induced single, discrete and uniform-sized lesions irrespective of the inoculum type (mature, immature sclerotium, and mycelium). Use of immature sclerotia is a simple, rapid, and highly reproducible disease production assay under greenhouse conditions (Singh *et al*., 2002).

Further studies indicated that the pathogen when inoculated on inner surface of rice sheath first colonized the surface before producing lobate, bulbous appressoria and infection cushions. The colonization of epidermal and mesophyll cells was both intra and inter-cellular. The intra-cellular hyphae were thick and deformed whereas the surface hyphae from primary lesions penetrated the healthy tissue both by hyphal tips as well as branched lobate appressoria. Early infection on a healthy plant within 12 h is possible when mycelium of the pathogen was used instead of sclerotial bodies (Singh *et al*., 2003).

The ShB pathogen produces several cell wall degrading enzymes (CWDEs) in improved Marcus medium under *in vitro* conditions. CWDEs include polygalacturonase (PG), cellulase (Cx), pectin methylgalacturonase (PMG), and polygalacturonic acid trans-eliminase (pectate lyase) (PMTE) in improved Marcus's medium of which the activity of PG, Cx, and PMG were significantly higher than PGTE and PMTE. These CWDEs play an important role in lesion formation and expansion (Chen *et al*., 2006). Rice ShB pathogen also produces toxin that induce characteristic symptoms on rice leaves, wilting of seedlings, and inhibited rice radicle growth. A positive correlation was noted between crude toxin production and the virulence of the pathogen (Xu *et al*., 2004).

2.3 Microorganisms in the management of plant diseases

Soil borne pathogens are complex not only in their behavioural pattern but also in their biochemical constituents. Hence, it is not very easy to control these pathogens.

Understanding and dealing with soil borne pathogens is a very difficult and a challenging task (Dev and Dawande, 2010).

To date, many promising microbes have been reported as biocontrol candidates against soilborne and airborne fungal plant pathogens. Most studies have emphasized fungal and bacterial agents such as *Trichoderma* sp. (Green *et al*., 1999), *Pseudomonas* spp. (Maurhofer *et al*., 1995; Murakami *et al*., 1997; Rodriguez and Pfender, 1997). *Agrobacterium* sp. (Penyalver and Lapez, 1999), *Stenotrophomonas maltophilia* (Zhang and Yuen, 1999), *Serratia marcecens* (Iyozumi *et al*., 1996 and Okamoto *et al*., 1998) and actinomycetes spp. (Shimizu *et al*., 2000).

2.3.1 *Trichoderma spp.*

Several strains of *Trichoderma* have been developed as biocontrol agents against fungal diseases of plants. The various mechanisms include antibiosis, parasitism, inducing host-plant resistance, and competition. Most biocontrol agents are from the species *Trichoderma harzianum*, *Trichoderma viride* and *Trichoderma hamatum* (Chet, 1987). The biocontrol agent generally grows in its natural habitat on the root surface, and so affects root disease in particular, but can also be effective against foliar diseases. *Trichoderma*, being a saprophyte adapted to thrive in diverse situations, produces a wide array of enzymes. By selecting strains that produce a particular kind of enzyme, and culturing these in suspension, industrial quantities of enzyme can be produced. *Trichoderma* spp. are highly efficient producers of many extracellular enzymes such as beta 1, 4 glucanase, beta 1, 3 glucanase, chitinases (Chet, 1990). They are used commercially for production of cellulases and other enzymes that degrade complex polysaccharides (Dev and Dawande, 2010).

2.3.2 *Pseudomonas fluorescens*

Pseudomonas fluorescens has multiple flagella. It has an extremely versatile metabolism, and can be found in the soil and in water. It is an obligate aerobe but certain strains are capable of using nitrate instead of oxygen as a final electron acceptor during cellular respiration. Certain *Pseudomonas fluorescens* isolates produce the secondary metabolite 2, 4 diacetylphloroglucinol (2, 4-DAPG), the compound found responsible for antiphytopathogenic and biocontrol properties in these strains. The *phl* gene cluster encodes factors for 2, 4-DAPG biosynthesis, regulation, export, and degradation (Dev and Dawande, 2010).

2.3.3 Pink-pigmented facultative methylotrophs (PPFM)

Methylobacterium spp. are a group of bacteria known as pink-pigmented facultative methylotrophs, or PPFMs (Austin and Goodfellow, 1979; Patt *et al.*, 1976; Green and Bousfield, 1982, 1983). They are classified as alpha-*Proteobacteria* and are capable of growth on one-carbon compounds such as formate, formaldehyde, methanol, and methylamine as well as on a variety of C_2, C_3 and C_4compounds (Lidstrom, 2001). Pink-pigmented facultative methylotrophs can induce various defense related proteins and synthesis of phenolics. The defense related enzymes, including PR-proteins, are reported to accumulate in plants with the onset of induced systemic resistance in presence of methylotrophs against sheath blight of rice causing by *R. solani* (Madhaiyan *et al.*, 2006).

2.3.4 Actinomycetes

Actinomycetes, especially *Streptomyces* spp., isolated from the rhizosphere have been the focus as excellent biocontrol agents to soilborne plant diseases. Such effective activity is largely dependent on secondary metabolites produced by these organisms. *Streptomyces* spp. is capable of producing a remarkably wide spectrum of antibiotics as secondary metabolites. Knowledge about the novel antibiotics produced by *Streptomyces* spp. is still accumulating. For example, in the last two decades novel antibiotics produced by *Streptomyces* spp., such as alnumycin, phthoxazolin A, phthoxazolin B-D, polyene antibiotics (AB023), vinylamycin and geldanamycin, were discovered. Of these antibiotics, antagonistic activities against several phytopathogenic fungi have been shown for AB023 and phthoxazolin B-D (Shimizu *et al.*, 2000).

2.4 Evaluation of bioagents against *Rhizoctonia solani*

2.4.1 *In vitro* evaluation of bioagents

Ramanathan *et al.* (2002) stated that actinomycetes spp. as biological control is slow but can be long lasting, inexpensive, and harmless to living organisms and the ecosystem; it neither eliminates the pathogen nor the disease, but brings them into natural balance.

Bressan *et al.* (2003) observed that actinomycetes are known to produce bioactive substances, especially antibiotics that are effective against phytopathogenic fungi.

Cao *et al.* (2004) isolated endophytic streptomycete isolates and reported that twenty-one per cent of endophytic streptomycete isolates produced antibacterial metabolites and 41 per cent produced antifungal metabolites in S medium. Sixty-five per cent of the most frequently isolated strains inhibited the growth of *Rhizoctonia solani* by the antibiosis assay but only 32 per cent produced metabolites active against *R. solani* in S medium. Growth promotion and enhanced disease resistance of seedlings inoculated with Streptomyces sp. Strain S30 were observed in tomato.

Kamalakannan *et al.* (2004) evaluated five isolates each of *Trichoderma viride* and *Pseudomonas fluorescens* and four isolates of *Bacillus subtilis* for their ability to control *Rhizoctonia solani*. Among the isolates tested, TVUV10 (*T. viride*), PFMMP (*P. fluorescens*) and BSG3 (*B. subtilis*) showed maximum *in vitro* inhibition of mycelial growth of *R. solani*. Isolate PFMMP was also very effective in reducing disease incidence in greenhouse conditions.

Compant *et al.* (2005) studied that *Pseudomonas cepacia* synthesizes β-1,3-glucanase that destroys the integrity of *R. solani*, *S. rolfsii*, and *Pythium ultimum* cell walls.

Vaish *et al.* (2006) obtained thirteen isolates of *Gliocladium spp* and four isolates of *Aspergillus niger* from different sources *viz.*, soil, rice rhizosphere, rice leaves and rice leaf sheath were evaluated for their biocontrol ability against *R. solani*, the causal agent of sheath blight of rice. Four isolates of *Gliocladium* spp. and 4 isolates of *A. niger* were found effective in dual culture. Hyphal interactions between biological control agents and *R. solani* were observed as hyperparasitic coiling, penetration and finally resulting lysis and collapse of *R. solani*. Two isolates of *Gliocladium* spp. and all four isolates of *A. niger* were found highly inhibitory to the mycelial growth of the pathogen by producing volatile substances. Culture filtrate of the promising isolates of antagonists were also inhibited the growth of the pathogen.

Rini and Sulochana (2007) observed that the general inadequacy of chemical fungicides to tackle *Rhizoctonia solani* and *Fusarium oxysporum* diseases in tomato has led to the search for biocontrol solutions to these maladies. Twenty-six local isolates of *Trichoderma* spp. and 56 isolates of fluorescent pseudomonads from Kerala were evaluated for their antagonistic activity against *R. solani* and *F. oxysporum* under *in vitro* conditions. Different isolates showed varying degrees of antagonism. The two most antagonistic isolates against *R. solani* were *T. pseudokoningii* TR17 and *T. harzianum* TR20. Likewise, *T. viride* TR19 and TR22 formed the most effective isolates against *F. oxysporum*. Production of volatile and non volatile antibiotic compounds varied among these isolates. Of the fluorescent pseudomonads, *Pseudomonas fluorescens* isolates P28 and P51 showed the greatest inhibition against *R. solani* whereas against *F. oxysporum*, P20 and P28 were most effective.

Islam *et al.* (2009) isolated *Streptomyces albidoflavus* C247 from the soil and analysed it on physiological, biochemical and 16S rDNA gene sequence analysis which strongly suggested that the isolate belonged to *Streptomyces albidoflavus*. Preliminary screening revealed that the isolate was active against fungal and bacterial pathogen. Additionally, 62.89 per cent mycelial growth inhibition was achieved against fungal pathogen. Ethyl acetate (EtOAc) was the best extraction solvent for the isolation of the antibiotics, and 100 µg/ml of EtOAc extract was found to inhibit 60.27 per cent of the mycelial growth of *Rhizoctonia solani* AG2-2 (IV).

Ningthoujam *et al.* (2009) obtained several actinomycete isolates from various habitats in Manipur were screened for activity against some major rice fungal pathogens showed potent antagonistic activities in dual culture assay.

Vijay Krishna Kumar *et al.* (2009) studied seventy rhizobacterial isolates of *Bacillus*, *Brevibacillus*, *Paenibacillus* and *Arthrobacter* spp for antagonistic activity against mycelial growth, sclerotial germination and sheath blight lesion development on leaf blades under *in vitro* conditions, which revealed that the mycelial growth of *R. solani* was inhibited up to 83 per cent by these PGPR and 10 strains were found to exhibit antagonism of over 70 per cent.

Amin *et al.* (2010) tested six isolates of *Trichoderma* spp. for their ability to produce volatile metabolites against seven fungal plant pathogens *viz.*, *Fusarium oxysporum* (causing chilli wilt), *Rhizoctonia solani* (causing sheath blight of rice), *Sclerotium rolfsii* (causing collar rot of tomato), *Sclerotinia sclerotiorum* (causing web blight of beans), *Colletotrichum capsici* (causing anthracnose of chilli fruit), *Helminthosporium oryzae* (causing brown spot of rice), *Alternaria brassicicola* (causing Alternaria blight of cabbage). Studies indicated that *T. viride* (Tv-1) was most effective in reducing the mycelial growth of *F. Oxysporum* (41.88%), whereas, in case of *R. solani* *T. viride* (Tv-2) accounted for maximum reduction in mycelia growth (30.58%) and sclerotial production (65.65%).

Amin *et al.* (2010) tested six isolates of *Trichoderma* spp. for their ability to inhibit soil borne pathogens of different vegetables *viz.*, *Rhizoctonia solani* (isolates from tomato), *Sclerotium rolfsii* (causing collar rot of tomato) and *Sclerotinia sclerotiorum* (causing web blight of beans) under *in vitro* conditions. Dual culture of pathogens and *Trichoderma* spp. revealed *T. viride* (Tv-2) highly inhibited the mycelial growth (71.41 per cent over control) in case of *Rhizoctonia solani* followed by *T. viride* (Tv-1) and *T. harzianum* (Th-1) showing 65.71 and 60.51 per cent inhibition over control, respectively.

Similarly in case of *Sclerotium rolfsii* and *Sclerotinia sclerotiorum, T. viride* (Tv-1) proved to be best overall isolates in inhibiting mycelial growth of test pathogens (67.91 and 66.21 per cent inhibition over control, respectively).

Dev and Dawande (2010) found that the diseases caused by soil borne plant pathogen *Rhizoctonia solani* can be controlled by the antifungal activity of *Trichoderma* spp. and *P. fluorescens*. These two antifungal agents produces wide variety of enzymes such as beta 1, 4 glucanase, beta 1, 3 glucanase, chitinases *etc*., Thus their study established the antagonistic property of *Trichoderma* spp. and *P. fluorescens* against *Rhizoctonia* sp. *i.e.* a plant pathogen.

Khair (2010) studied the inhibitory effect of *T. album, T. hamatum*, *T. harizianum* and *T. viride* against the mycelia growth of *F. solani* and *R. solani in vitro* test. The antagonistic effects of *Trichoderma* spp. against *F. solani* were in the range of 38.9 – 57.5 per cent. *T. hamatum* gave the highest effect about 57.5 per cent, followed by *T. album* (49.1%), *T. harzianum* (44.4%) and *T. viride* (38.9%), respectively. Results showed that the growth inhibition of *R. solani* by *Trichoderma* spp. were in the range of 41.7 – 70.3 per cent. *T. hamatum* also highly inhibited the mycelial growth of *R. solani*, where the growth inhibition was 70.3 per cent, followed by *T. harzianum* (61.1%), *T. album* (53.7%) and *T. viride* (41.7%), respectively. Results showed that the best growth inhibition against two pathogenic fungi was obtained by *T. hamatum*, while the lowest one was obtained by *T. viride*.

Reddy *et al.* (2010) studied eight *Pseudomonas fluorescence* strains against *R. solani*. All the strains tested were exhibited antagonistic activity against *R. solani*. One isolate, Pf 003 gave 78 per cent inhibition compared to control. All the fungicides and crude extracts of Pf 003 inhibited the mycelial growth of *R. solani*. Highest inhibition was recorded with hexaconazole and ethyl acetate crude metabolite extract. The results offer a scope for integrating *P. fluorescens* with chemical fungicides for the control of sheath blight of rice.

Al-Askar *et al.* (2011) isolated one hundred and twenty-eight strains of actinomycetes from different soils in Riyadh region, Saudi Arabia and screened for their antifungal activities against *R. solani*, *Fusarium solani*, *F. verticillioides, Alternaria alternata* and *Botrytis cinerea.* A potent antagonist against all tested phytopathogenic fungi was selected and identified as *Streptomyces spororaveus.*

Patale and Mukadam (2011) tested the antagonistic activities of three *Trichoderma* species, *i.e. T. viride*, *T. harzianum*, and *Trichoderma* sp. against seven pathogenic fungi, namely *Aspergillus niger*, *A. flavus, Phytophthora* sp., *Fusarium oxysporum*, *Rhizoctonia solani*, *Penicillium notatum*, and *Alternaria solani*. They found that all three species of *Trichoderma* effectively suppressed the growth of seven pathogenic fungi.

Priya and Kalaichelvan (2011) obtained several actinomycete isolates from various habitats in Tamil Nadu and screened for activity against some major rice fungal pathogens in dual culture assay. The nature of the activity in terms of fungitoxic or fungistatic nature was also determined.

Al-Askar (2012) carried out the study with the isolation of *Streptomyces* bacteria from soil samples of Dilam city. Seventy two (72) *Streptomyces* isolates were isolated and screened against six different fungal phytopathogen, out of seventy two, twenty four was shown to possess the highest antifungal activity especially against 6 fungi, *Fusarium* sp., *Fusarium* sp., *Macrophamina* sp., *Macrophamina* sp., *Rhizoctonia* sp. and *Fusarium* sp.

Mohpatra *et al.* (2012) isolated two hundred and forty eight strains of actinomycetes from different soils in Western Ghats and one hundred and forteen isolates screened against phytopathogens *Rhizoctonia solani, Sclerotium rolfsii, Colletotrichum sp.*and plant pathogenic bacterium, *Ralstonia solanacearum*.

Mary and Dhanaseeli (2012) identified a potential actinomycete for the control of *Rhizoctonia solani.* The selected actinomycete was found to cause hyphal lysis in *Rhizoctonia solani* and inhibition of growth of mycelium by the production of toxic volatile and non-volatile metabolites and hydrolytic enzymes such as chitinase and β-1,4- endoglucanase.

Srividya *et al.* (2012) identified a potent actinomycete isolate 9p with broad spectrum antifungal property against four phytopathogens *Alternaria brassiceae* OCA3; *Collectotrichum gleosporioides* OGC1; *Rhizoctonia solani* MTCC 4633 and *Phytophthora capsici*. And also different isolates were identified as *Streptomyces sp.* based on colony morphology, microscopy and 16srDNA. Further study of isolate showed 87.5 per cent germination index when coated onto chilli seeds. This coupled with antifungal properties, suggests both the PGPR and biocontrol aspect of the actinomycete *Streptomyces* sp.

2.4.2 *In vivo* evaluation of bioagents

Elad *et al.* (1980) observed that under greenhouse conditions, incorporation of the wheat-bran inocula preparation of *T. harzianum* in pathogen-infested soil reduced significantly bean diseases caused by *S. rolfsii*, *R. solani*, or both, but its biocontrol capacity was inversely correlated with temperature. The wheat-bran preparation of *T. harzianum* increased growth of bean plants in a non-infested soil and it controlled *S. rolfsii* more efficiently than a conidial suspension of the same antagonist. In naturally infested soils, wheat-bran preparation of *T. harzianum* inocula significantly decreased diseases caused by *S. rolfsii* or *R. solani* in three field experiments with bean, cotton, or tomato, and these increased significantly the yield of beans.

Venkatasubbaiah *et al.* (1984) studied the effectiveness of an antagonistic isolate, *Trichoderma harzianum* Rifai for controlling collar rot of coffee seedlings. Under field conditions using natural inocula of *Rhizoctonia solani* Kuhn and under artificially infested soils, seed treatment with *T.harzianum* brought down the incidence of collar-rot disease. A soil incorporation method of inocula was superior to seed treatment. The possibility of using *T.harzianum* as a biocontrol agent against collar-rot of coffee is discussed.

Windham *et al.* (1986) studied that *Trichoderma* spp. produced a growth regulating factor that increases the rate of seed germination and dry weight of shoots of rice. Chet (1987) studied that the genus *Trichoderma* is especially known for its antagonistic activity against several plant pathogens, including *R. solani.*

Thomashow and Weller (1988) reported that production of antibiotics, iron chelating siderophores, cyanide and lytic enzymes by soil-residing bacteria are the major contributing factors in the biocontrol against pathogens by inhibiting both the germination and growth of their survival structures and mycelium in the rhizosphere and spermosphere of plants.

Groth *et al.* (1990) studied that some potentially effective fungicides are highly phytotoxic to rice and, if the disease is not severe, these fungicides may reduce yield. Mostafa Kamal and Shahjahan (1995) studied that *Trichoderma* spp. are also potential agents in suppressing rice sheath blight, they are highly competitive on rice plant residue and thus exhaust the nutrient supply for the pathogen and greatly reduce its survival.

Use of chitinase-producing *Streptomyces* spp. and *Bacillus cereus* isolates used in combination with antibiotic producing *P. fluorescens* and *Burkholderia* (*Pseudomonas*) *cepacia* isolates have shown a synergistic effect on the suppression of rice sheath blight incited by *Rhizoctonia solani* (Sung and Chung, 1997).

In rice, seed-treatment and root-dipping of rice seedling with PGPR strain mixtures, *viz.*, *P. fluorescens* strains Pf1 and PB2 reduced rice sheath blight disease incidence and improved the grain yield under field condition (Nandakumar, 1998).

Strains of *T. viride* and *T. harzianum* have been reported to efficiently control *R. solani* in rice (Krishnamurthy *et al.*, 1999). Das and Hazarik (2000) observed that it is difficult to achieve control on sheath blight through host resistance or fungicides, therefore, biological control may be effective in minimizing the incidence of sheath blight.

De Vleesschauwer *et al.* (2006) reported that root colonization of rice by *Pseudomonas aeruginosa* 7NSK2 renders foliar tissues more resistant to infection by *Magnaporthe oryzae*.

Prabavathy *et al.* (2006) studied effect of antifungal aliphatic compound of *Streptomyces sp.* PM5 (SPM5C-1) at greenhouse experiments which revealed that spraying of SPM5C-1 on rice significantly decreased blast and sheath blight development by 76.1 per cent and 82.3 per cent, respectively, as compared to the control with a corresponding increase in rice grain yield.

Rini and Sulochana (2006) studied isolates of *Trichoderma* (*T. harzianum* TR20 and *T. pseudokoningii* TR17) and fluorescent pseudomonads (*Pseudomonas fluorescens* P28 and P51) were evaluated (alone and in combination) under greenhouse and field conditions for efficacy in suppressing rhizoctonia root rot incidence and promoting plant growth in chilli. The combination, *T. harzianum* (TR20) + *P. fluorescens* (P28), was most effective in reducing disease incidence (66.7 per cent more efficient than the control), but was at par with copper oxychloride (0.3%).

Highest per plant yield also was recorded in the treatment combination TR20 + P28, followed by *T. pseudokoningii* (TR17) + *P. fluorescens* (P51). *T. pseudokoningii* (TR17) and *T. harzianum* (TR20) when applied alone also significantly increased the yield per plant and was superior to both the pseudomonads applied individually.

Dawar *et al.* (2008) tested the biocontrol potential of different microbial antagonists, *i.e. Bacillus thuringiensis*, *Rhizobium meliloti*, *Aspergillus niger* and *Trichoderma harzianum* by coating the seeds with gum Arabic, glucose, sucrose and molasses. This method reduced successfully the infection of *Macrophomina phaseolina*, *Rhizoctonia solani* and *Fusarium* spp. The highest suppression capacity was shown by seed treatment with *T. harzianum* using 2 per cent of glucose.

The enhancement of rice growth was influenced by the plant growth promoting bacteria (PGPB) collected from the rhizosphere soil of rice field (Ashrafuzzaman *et al.*, 2009).

Fatima *et al.* (2009) were able to isolate plant growth promoting bacteria (PGPB) strains that positively affected the germination of wheat, increased biomass and root shoot length by inhibiting *R. solani* growth.

Khair (2010) observed in greenhouse experiment that *T. album, T. hamatum*, *T. harizianum* and *T. viride*, as soil treatments, significantly reduced the pre- and post-emergence damping off disease incidence under artificial infection with *F. solani* and *R. solani*. Soil treatments with four *Trichoderma* species significantly reduced the incidence of damping off disease, where the percentages disease incidence were in the range of 7.0 -20.0 per cent and 2.4 – 6.5 per cent, compared to 25.7 and 13.5 per cent in control plants, at pre- and post-emergence stages, respectively.

Lanwongsa (2010) identified *Pseudomonas* biocontrol strains produce anti-fungal metabolites, of which DAPG, phenazines, pyrrolnitrin, pyoluteorin and volatile hydrogen cyanide are the most frequently detected classes.

Anitha and Das (2011) isolated *Pseudomonas fluorescens* and *Trichoderma* sp.from rhizosphere soil and the antagonistic activity of isolates was observed against *Rhizoctonia solani*. Biological control and hormonal inducers represents an interesting strategy against pathogen especially when applied together. Rice plants infected with *R. solani* were treated with biocontrol agents along with arbuscular mycorrhizal (AM) fungi and/or sprayed with hormonal inducers (Salicylic acid). Plants treated with biocontrol agents alone showed moderate growth. Likewise VAM alone treated plants showed very good result whereas combination of VAM and salicylic acid did not show considerable growth response.

Prasad *et al.* (2011) evaluated three isolates of *Trichoderma* spp. against sheath blight disease of rice and found that among the three one isolate (*Trichoderma* spp. TN3) was most effective in reducing disease incidence and increasing grain yield. Though the disease incidence was more on TN3 treated plants than hexaconazole treatment, the increase in grain yield was almost same in both the treatments. The bioagent could effectively controlled the disease and at the same time improved growth characters under the glasshouse conditions.

2.5 Delivery systems

Plant growth promoting rhizobacteria are delivered through several means based on survival nature and mode of infection of the pathogen.

2.5.1 Seed treatment

In rice, seed treatment with PGPR strains increases the chitinase enzyme activity and phenolic content and this is correlated with the reduced nematode infestation (Swarnakumari, 1996).

Vidhyasekaran and Muthamilan (1999) studied rice seed-treatment followed by root-dipping and a foliar spray with *P. fluorescens* strains Pf1 and FP7 showed higher induction of ISR against the sheath blight pathogen, *Rhizoctonia solani.*

Soaking of rice seeds in water containing 10 g of talc based formulation of *P. fluorescens* consisting of PF1 and PF2 (10^8 cfu/g) for 24 h controlled rice sheath blight under field condition (Nandakumar *et al.*, 2001).

Sharma *et al.* (2004) showed that seed treatment with antagonistic bacteria like PGPR, fluorescent *Pseudomonads and Bacillus spp* controlled sheath blight as well as resulted in increased root and shoot length of rice seedlings.

Madhaiyan *et al.* (2006) studied plant growth-promoting activity of methylotrophic bacteria and their effects on disease suppression were evaluated on rice under greenhouse conditions where rice seeds were inoculated with *Methylobacterium* sp. strain PPFM-Os-07 and the results suggested that *Methylobacterium* inoculation may alter rice susceptibility to *R. solani*.

2.5.2 Soil application

Nandakumar *et al.* (2001) broadcasted the talc based formulation of strain mixtures of *Pseudomonas* spp. (Pf1 and FP7) by blending 2.5 kg of formulation with 50 kg of sand after 30 days of transplanting paddy seedlings to main field significantly reduced sheath blight and increased yield under field, conditions.

Effective ShB disease suppression at field level can be obtained by soil application of *T. harzianum* and *T. viride* at a pH range of 5.1 to 6.0. A concomitant increase in plant growth and yield was obtained. Further, it was reported that population levels of *Trichoderma* spp are high and that of *R. solani* are low in acid soils (Bhagawati, 2005).

Khan *et al.* (2005) examined that increased seedling emergence, root and shoot length, fresh and dry weight of root of rice seedlings, as compared to check when bioagent *Trichoderma harzianum* was applied as soil treatment with the bioagent @ 8 gm/kg soil.

Rajendran and Samiyappan (2008) studied the efficacy of talc based bioformulation of *Pseudomonas fluorescens* through soil application against *R. solani*, which minimize the disease incidence compared to control.

2.5.3 Foliar spraying method

Lumsden *et al.* (1995) studied the many solid or powder formulations can also be made into liquid- or water-based suspensions to fulfill the delivery requirements of drench, spray or dip treatments of plant growth promoting rhizobacteria(PGPR), in further studies which showed that foliar spraying gave highest reduction of disease symptoms of fungal phytopathogens.

Singh and Sinha (2005) investigated the effectivity of potential strains of *Pseudomonas fluorescens* against sheath blight of rice under field conditions, applied as three time sequences. Foliar sprays with Pfr 1, 7 days before pathogen inoculation resulted in maximum reduction in sheath blight severity (59.6-64.4) and incidence (36.7-40.4), and increasing in grain yield (30.6-32.3) and 1000-grain weight (27.2 29.5). Simultaneous application of the bioagent (s) and *R. solani* or bioagents applied 7 days after inoculation with *R. solani* was relatively inferior in reducing the disease.

Foliar application of *Trichoderma* spp also was found to be very effective in reducing ShB severity. Studies on field application of *T. harzianum* as talc + CMC based formulation proved that disease severity was reduced by 52 per cent. The bioagent was found effective, when applied at 7 days compared to simultaneous application with ShB pathogen (Khan and Sinha, 2006)

Mode of application of the bacterial bioagent and the type of formulation also affects its efficacy under greenhouse and field conditions (Kanjanamaneesathian *et al.,* 2007). Floating pellet and water-soluble granule formulations of *B. megaterium* were found effective against rice ShB disease. Of these, foliar spraying of the bioagent was more effective than the floating pellet formulation in reducing the percent ShB affected tillers.

Manonmani *et al.* (2008) studied bacterial biocontrol agents *viz., Pseudomonas fluorescens* (Pf1) and *Bacillus subtilis* have been formulated in different carriers *viz.,* gypsum, peat soil and talc powder. The results revealed that gypsum based formulation of two bioagents were sprayed on diseased rice crop which was effective in controlling the disease by recording the disease incidence of 10.67 per cent and 12.00 per cent respectively as compared with unsprayed plants exhibited the maximum disease incidence of 90.94 per cent.

2.5.4 Combination of delivery systems

In rice, seed-treatment with *P. fluorescens* strain Pf1 induces resistance which is observed upto 45 days after sowing. When a foliar spray is given at 45 days after sowing, the resistance is observed at 4 days after application and persists for 15 days in rice leaves (Vidhyasekaran *et al.*, 1997). Foliar sprays of *P. fluorescens* formulations should be given at every 15 days intervals for managing rice foliar diseases. A parallel experiment conducted by Nayar (1996) indicated that induction of defence mechanisms using *P. fluorescens* persisted upto 60 days by seed-treatment, 30 days by root-dipping and 15 days by foliar spray against rice blast.

Seed treatment of pigeonpea with talc based formulation of fluorescent *Pseudomonas* @ 4 g per kg of seed followed by soil application at the rate of 2.5 kg/ha at 0, 30 and 60 days after sowing controlled pigeonpea wilt incidence under field conditions. The additional soil application of talc based formulation improved disease control and increased yield compared to seed treatment alone (Vidhyasekaran *et al.*, 1997).

Delivering of *Pseudomonas fluorescens* as seed treatment followed by three foliar applications suppressed rice blast under field conditions (Krishnamurthy and Gnanamanickam, 1998).

The increased efficacy of strain mixtures through combined application might be due to increase in the population of fluorescent *Pseudomonas* in both rhizosphere and phyllosphere (Viswanathan and Samiyappan, 1999).

Combined application of talc based formulation of fluorescent *Pseudomonas* comprising of Pf1 and FP7 through seed treatment, seedling dip, soil application and foliar spray suppressed rice sheath blight and increased plant growth better than application of the same strain mixture either through seed, seedling dip or soil (Nandakumar *et al.*, 2001).

Seed and foliar application of fluorescent *Pseudomonas* reduced leaf spot and rust of groundnut under field conditions (Meena *et al.*, 2002).

Prasad *et al.* (2003) tested *Pseudomonas fluorescens* PDBCAB19 and *P. putida*, PDBCAB 2 under field conditions against blight of sunflower caused by *Alternaria helianthi*. Blight incidence with respect to plant growth promoting rhizobacteria and fungicide treatments alone or in combination treatments was significantly lower and disease severity ranged between 35.4 and 65.2 per cent compared to the pathogen check (86.4%).

Ramesh and Korikanthimath (2003) evaluated two isolates of *Pseudomonas fluorescens* against *Pythium aphanidermatum* (damping off agent) and *Ralstonia solanacearum* (wilt) in brinjal. Application of these biocontrol agents as seed and soil treatments reduced both pre and post-emergence damping off, ranging from 18 to 42 per cent and 23 to 55 per cent respectively. The growth parameters and vigour index recorded in nursery were higher in *P. fluorescens* treatments. The least population of the pathogen was observed after 20 days of treatment.

Singh (2003) used fluorescent *Pseudomonas* isolated from rhizosphere and phyllosphere of rice, chickpea, chilli, brinjal and soil samples. The antagonists were applied as root dip method, seed treatment and combination of soil plus root dip method. The combination treatment showed maximum protection against *Rhizoctonia solani, S. sclerotium, M. phaseolina, F. oxysporum* and *P. vexans* on three vegetables as compared to single treatment alone. The soil + root dip treatments with isolate C7 (French isolated) gave maximum inhibition of disease *i.e.*, 73.4, 61.2, 77.8, 75.00, 85.4, 90.0 per cent caused by *R. solani* (root rot of chilli). *M. phaseolina* (charcoal rot of chilli) *Sclerotium spp.* (stem rot of tomato) and *P. vexans* (Phomopsis blight of brinjal) respectively.

Application of strain mixture based formulation of Pf1 and FP7 with or without chitin through seed, seedling dip and foliar spray suppressed leaf folder damage and sheath blight in rice under field conditions (Radjacommare *et al.*, 2004).

Sajeena *et al.* (2004) evaluated biocontrol agents in pathogen infested soil in pots for suppression of dry-rot pathogen in blackgram. Seed treatment with biocontrol agents was found to be more effective than their respective soil application. Population of biocontrol agents in blackgram rhizosphere was directly related to suppression of the dry root rot pathogen (*Macrophomina phaseolina*).

Singh *et al.* (2004) used *Trichoderma viridae, T. harzianum, Gliocladium virens* and *Aspergillus nidulans* and evaluated them as seed, soil and combined seed and soil treatments for control of tomato wilt (*Fusarium oxysporum*) in greenhouse. Seed treatment @ 10 g per kg seed was effective in controlling seedling mortality upto 85 per cent.

Lakshmi and Rajbir (2005) evaluated different delivery systems for *Trichoderma harzianum*. The combination of soil treatment, root dip and foliar spray together was found to be the most effective method and showed significantly reduced sheath blight disease severity in rice (29.07%) and disease incidence (58.40%) as compared to other treatments.

2.6 Molecular mechanism involved in interaction

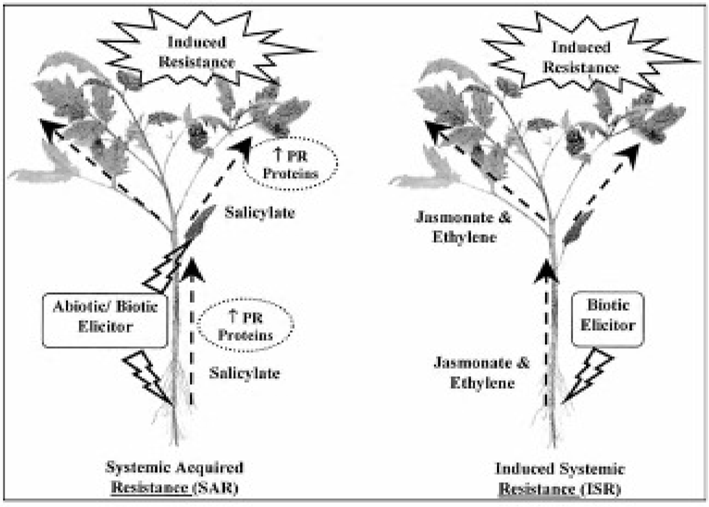


Fig.1. A pictorial comparison of the two best characterized forms of induced resistance in plants, both of which lead to similar phenotypic responses. Systemic acquired resistance, induced by the exposure of root or foliar tissues to abiotic or biotic elicitors, is dependent of the phytohormone salicylate (salicylic acid), and associated with the accumulation of pathogenesis-related (PR) proteins. Induced systemic resistance, induced by the exposure of roots to specific strains of plant growth-promoting rhizobacteria, is dependent of the phytohormones ethylene and jasmonate (jasmonic acid), independent of salicylate, and is not associated with the accumulation of PR proteins (or transcripts)(Vallad and Goodman,2004).

2.6.1 Plant signaling pathways induced by bioagent leading to disease resistance

Contact with pathogenic and nonpathogenic microorganisms triggers a wide range of defense mechanisms in plants. Two main mechanisms are recognized: systemic acquired resistance (SAR) and induced systemic resistance (ISR).

SAR is usually triggered by local infection, provides long-term systemic resistance to subsequent pathogen attack, is correlated with the activation of PR genes, and requires the involvement of the signal molecule salicylic acid (SA) (Durrant *et al.*, 2004).

ISR is known to result from colonization of roots by certain nonpathogenic rhizosphere bacteria (Van Loon *et al.*, 1998). ISR is not SA-dependent, but rather requires components of the jasmonic acid (JA) signaling pathway followed by the ethylene signaling pathway.

Interestingly in this regard, a 17-year-old report of systemic resistance in rice (*Oryza sativa*) triggered by preinoculation with an hypersensitive response (HR) -eliciting, nonpathogenic *Pseudomonas syringae* strain remains to be one of the most compelling examples of a monocot SAR-like response to date (Smith and Metraux, 1991).

Application of *Trichoderma harzianum* to bean roots resulted in a 25 to 100 per cent reduction in the severity of foliar grey mold, caused by *Botrytis cinerea.* Biocontrol fungus *T. harzianum* T39 and a chemical BTH (benzothiadiazole) were tested for induction of resistance in tomato to *B. cinerea*. In these experiments, it became obvious that resistance-inducing strains stopped the fungus at the early stage and the number of spreading lesions declined to about 30 per cent (Audenaert *et al.*, 1998), indicating systemically induced resistance.

Krishnamurthy and Gnanamanickam (1998) described ISR in rice in response to inoculation with different strains of plant growth–promoting Pseudomonas as an important mechanism in the biological suppression of sheath blight and blast disease of rice.

Bailey and Lumsden (1998) studied that specific strains of fungi in the genus *Trichoderma* colonize and penetrate plant root tissues and initiate a series of morphological and biochemical changes in the plant, considered to be part of the plant defense response, which subsequently leads to induced systemic resistance.

Hua *et al.* (1998) reported ethylene response is considered to be downstream of JA response in rhizobacteria-mediated ISR. ETR1 and CTR1 proteins work together to negatively regulate the ethylene response pathway in the absence of ethylene. De Meyer *et al.* (1999) studied that *Pseudomonas aeruginosa* 7NSK2 and *Trichoderma harzianum* T39 induced systemic resistance against *B. cinerea* on bean and tomato and stopped spread of the pathogen at a very early stage. However, when the infection pressure was very high, *B. cinerea* spread could not be controlled effectively by induced resistance.

Yedidia *et al.* (1999) suggested that the cells of the biological control agent applied to the roots and dead cells applied to the leaves of cucumber plants induced control of powdery mildew. The activation of plant defense systems in association with roots treated with *T. harzianum* strain T 203, which also exhibited higher activities of chitinase, *ß*-1, 3-glucanase, cellulase and peroxidase when compared to an untreated control 72 hours post inoculation. Scanning electron microscopy revealed typical fungal structures previously associated with mycoparasitic interactions of *Trichoderma* spp. Treatment of cucumber plants with 2, 6- dichloroisonicotinic acid, an inducer of the plant defense response, displayed responses that were similar, but not identical to those plants inoculated with *T. harzianum.*

Khan *et al.* (2001) reported that *T. hamatum* 382 (T382) induced systemic resistance in cucumber against *Phytophthora* root and crown rot as well as leaf blight.

Pieterse *et al.* (2001) selected strains of rhizosphere bacteria to reduce disease by activating a resistance mechanism in the plant named rhizobacteria-mediated induced systemic resistance (ISR). Rhizobacteria-mediated ISR resembles pathogen induced systemic acquired resistance (SAR) in that both types of induced resistance render uninfected plant parts more resistant towards a broad spectrum of plant pathogens. Some rhizobacteria trigger the salicylic acid (SA) - dependent SAR pathway by producing SA at the root surface. In other cases, rhizobacteria trigger a different signaling pathway that does not require SA. The existence of a SA-independent ISR pathway has been demonstrated in *Arabidopsis thaliana* (Pieterse *et al.,* 2000).

In contrast to pathogen induced SAR, ISR induced by *Pseudomonas fluorescens* WCS417r is independent of SA accumulation and pathogenesis-related (*PR*) gene activation but, instead, requires responsiveness to the plant hormones jasmonic acid (JA) and ethylene.

Ton *et al.* (2001) studied rhizobacteria-mediated ISR in Arabidopsis, using *Pseudomonas fluorescens* WCS417r as the inducing agent and *P. syringae* pv. *tomato* DC3000 (*Pst*) as the challenging pathogen. To investigate how far, expression of ISR depends on the level of basal resistance, 10 different Arabidopsis ecotypes were screened for their potential to express WCS417r-mediated ISR and basal resistance against *Pst*. Two Arabidopsis ecotypes, RLD and Wassilewskija (Ws), were found to be blocked in their ability to express ISR. This ISR noninducible phenotype correlated with a relatively low level of basal resistance against *P.syringae pv.tomato*. Genetic analysis of crosses between the ISR-inducible ecotypes Columbia (Col) and Landsberg *erecta* (L*er*), on the one hand, and the non-inducible ecotypes RLD and Ws, on the other hand, revealed that ISR inducibility and basal resistance against *Pst* were inherited as monogenic dominant traits that are genetically linked.

Several species of Pseudomonas, Serratia, and Bacillus are known to protect plants directly by either producing antimicrobial compounds or indirectly through ISR in plants (Zhang *et al.*, 2002and Ryu *et al.*, 2004).

Chen *et al.* (2005) studied that in roots Constitutive triple response 1 (*CTR1*) and Ethylene response 1 (*ETR1*) both genes are induced by *Trichoderma*, suggesting ethylene response is inhibited. This local silencing of plant defense response probably enables symbiotic interactions, as has been observed for other symbiotic systems. Down regulation of the PR protein, pathogenesis-related maize seed (PRMS), in roots by *Trichoderma* inoculation is in agreement with local silencing of defense to allow fungal growth into the roots.

Haas and Defaso (2005) and Harman *et al.* (2004) identified some strains of root-colonizing microorganisms as potential elicitors of plant host defense. For example, some biocontrol active strains of *pseudomonas sp.* and *Trichoderma sp.* are known to strongly induce plant host defense.

In other instances, inoculation with Plant Growth Promoting Rhizobacteria (PGPR) have been shown to be effective in controlling multiple diseases caused by different fungal pathogens, including anthranose (*Colletotrichum lagenarium).* A number of chemical elicitors of SAR and ISR such as salicylic acis, sidesphore, lipopolysaccharides and 2,3- butanediol may be produced by the PGPR strains upon inoculation (Van Loon *et al.,* 1998; Ryu *et al.,* 2004).

Moreover, reports on SAR or ISR like phenomena in monocots are scarce (Kogel and Langen, 2005).

A research work was carried by Saksirirat *et al.* (2005) to investigate effects of species of *Trichoderma* in induction of systemic resistance in tomato against Fusarium wilt disease. A significant reduction in symptoms was observed under field conditions.

Ran *et al.* (2005) and Djavaheri (2007) demonstrated that the ability of *P. fluorescens* WCS374r to mount ISR in rice against the leaf blast pathogen *M. oryzae* and provide evidence that this WCS374r-mediated ISR is based on pseudobactin-mediated priming for a pronounced multifaceted cellular defense response. Furthermore, we show that WCS374r-triggered ISR functions independently of SA accumulation but, unlike benzothiadiazole (BTH) -inducible resistance, requires intact responsiveness to ET as well as a functional octadecanoid pathway.

Shoresh *et al.* (2005) studied the molecular mechanisms activated by *T. asperellum* in cucumber. Colonization of *T. asperellum* on roots induces resistance to *Pseudomonas syringae* pv. *lachrymans* (*Psl*) on foliage. During the process of the *Trichoderma* interaction with the plant, SA content did not differ from that of control plants, even though *Psl* infection did increase salicylate concentrations.

Distefano *et al.* (2008) evaluated two transgenic lemon clones with the chit42 gene from *Trichoderma harzianum* were tested for resistance to fungal disease and expression level of defence-related genes the overexpression of the transgenic fungal gene enhanced by two-three folds transcript levels of genes associated with enhanced ROS production and ISR establishment, while the expression of native chitinase and glucanase genes involved in SAR was down-regulated.

Stein *et al.* (2008) showed that priming may not occur universally in plant-*Trichoderma* interactions. There is a striking similarity between plant response to *Trichoderma* spp. and *Piriformospora indica* (*P. indica*).

Recent studies showed that *P. indica* may induce ISR through the JA/ethylene signaling pathway as well. *P. indica* root colonization reduced powdery mildew infection in *Arabidopsis* wild type and NahG mutant (unable to accumulate SA). However, two jasmonate signaling mutants were fully compromised in *P.* indica–mediated powdery mildew resistance even though their root colonization level did not differ from control plants.

Downstream of NPR1, the ISR and SAR signalling pathways diverge because, unlike SAR, ISR is not accompanied by the concomitant activation of *PR* genes (Pieterse *et al*., 1996; VanWees *et al*., 1997, 1999). Instead, ISR-expressing plants are primed for enhanced expression of predominantly JA- and ET-regulated genes upon pathogen infection (Verhagen *et al*., 2004; Cartieaux *et al*., 2008).

Successful establishment of ISR depends on the recognition of bacterial elicitors by the plant roots. Over the past decade, myriad bacterial traits operative in triggering ISR have been identified. Examples include flagella, cell envelope components such as lipopolysaccharides, and secreted metabolites, including antibiotics, quorum-sensing molecules, cyclic lipopeptides, volatiles, and siderophores (Bakker *et al*., 2007;Tran *et al*., 2007).

However, despite the increasing amount of research devoted to the identification and characterization of bacteria-derived ISR elicitors, much remains to be discovered about how these determinants are perceived and ultimately give rise to ISR. Compared with the vast information available for dicotyledonous plants, our understanding of the molecular machinery governing induced resistance responses in monocotyledonous crops is still in its infancy. Evidence demonstrating that central components of the SAR pathway, such as NPR1, are conserved in cereals has only recently been presented (Chern *et al*., 2001, 2005; Shimono *et al*., 2007; Yuan *et al*., 2007).

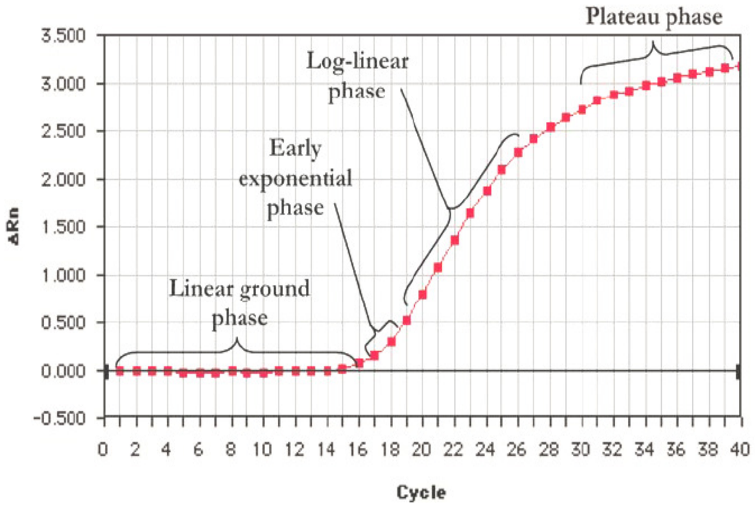
Mukherjee *et al.* (2012) provided evidence that PKS/NRPS hybrid enzyme is involved in *Trichoderma*-plant interactions resulting in induction of defense responses.

2.7 Expression profiling of defence genes

The study of gene function requires the ability to accurately quantify temporal and spatial patterns of gene expression. Traditional approaches, such as northern blots and RNase protection assays are in many cases unsuitable, as their low sensitivity required high concentrations of starting template (Sambrook and Russell, 2001). The advent of real-time PCR has enabled rapid and reproducible high throughput RT-PCR quantification, with an unparallel dynamic range and extremely high sensitivity. The first practical kinetic PCR technology, the 5'-nuclease assay was established in 1993 and combines the exponential PCR amplification of specific transcript with monitoring of newly synthesized DNA in each performed PCR cycle (Wong and Medrano, 2005). It is most sensitive method for the detection and quantification of gene expression levels, in particular for low abundant transcripts in tissues with low RNA concentrations, from limited tissue sample and for the elucidation of small changes in mRNA expression levels (Fleige *et al.*, 2006).

2.7.1 Quantitative real time reverse transcription PCR (qRT-PCR)

Real time quantitative PCR allow to sensitive, specific and reproducible quantitation of nucleic acid. A more accurate and faster method for determining of gene expression which require minimal amount of RNA is quantitative real time PCR (qRT-PCR), a fluorescence based reverse transcription polymerase chain reaction (Bustin, 2000). Real-time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. PCR can be broken into four major phases (Figure2): 1st phase is hidden under background fluorescence where an exponential amplification is expected; 2nd phase with exponential amplification that can be detected and above the background; 3rd phase linear amplification efficiency and a steep increase of fluorescence; and finally 4th phase or plateau phase, defined as the attenuation in the rate of exponential product accumulation, which is seen concomitantly in later cycles (Kainz, 2000). The amount of amplified target is directly proportional to the input amount of target only during the exponential phase of PCR amplification. Hence, the key factor in the quantitative ability of kinetic RT-PCR is that it measures the product of the target gene within that phase (Tichopad *et al.*, 2003). In plateau stage, reaction components become limited and the fluorescence intensity is no longer useful for data calculation (Bustin, 2000).



2.7.2 Relative quantification

Relative quantification determines the changes in steady-state mRNA levels of a gene across multiple samples and expresses it relative to the levels of an internal control RNA.

Relative quantification does not require standards with known concentrations and the reference can be any transcript, as long as its sequence is known. Ideally the reference gene should not be regulated or influenced by the experimental procedure. The accurate quantification of a true reference gene allows the normalization of differences in the amount of RNA or cDNA in individual samples generated due to the following reasons *i.e.* different amounts of starting material, the quality of the starting material, differences in RNA preparation and cDNA synthesis. Since, the reference gene is exposed to the same preparation steps as the gene of interest. It is crucial to choose the appropriate reference gene for an exact comparison of mRNA transcription in different samples or tissues (Radonic *et al.*, 2004; Dheda *et al.*, 2004; Silver *et al.*, 2006).

To calculate the expression of a target gene in relation to an adequate reference gene various mathematical models are established. Two types of relative quantification models are available:

(1) Without efficiency correction

$$\text{Ratio} = 2^{(\Delta \text{Ct sample} - \Delta \text{Ct control})}$$

(2) with kinetic pcr correction efficiency

$$\text{Ratio} = \frac{(\text{E target})^{\Delta \text{Ct (control – sample)}}}{(\text{E ref})^{\Delta \text{Ct (control – sample)}}} \div \frac{(\text{E ref})^{\text{Ct control}}}{(\text{E target})^{\text{Ct control}}}$$

Amplification efficiency of the reaction is an important consideration when performing relative quantification. Earlier methods of calculating gene expression have assumed the amplification efficiency of the reaction as ideal or 1; means the PCR product concentration doubles during every cycle within the exponential phase of the reaction (Gibson *et al.*, 1996). However, many PCR reactions do not have ideal amplification efficiencies, and calculations without an appropriate correction factor may overestimate starting concentration (Liu and Saint, 2002). When used appropriately, relative quantification can generate useful and biologically relevant information (Pfaffl, 2004).

2.7.3 Normalisation strategies

Quantitative real-time PCR (qRT-PCR) is today the prime technique to measure gene expression (Kubista *et al.,* 2006). When properly used it offers unprecedented sensitivity, accuracy and reproducibility. But there are caveats. The target is mRNA, which must be extracted and converted to cDNA in a reverse transcription process that can proceed at highly variable yield depending on the protocol. (Stahlberg *et al.*, 2004).

RNA is further rapidly degraded by nucleases abundant in biological samples (Pfaffl, 2006). To account for processing variation the expression of marker genes is normalized with appropriate endogenous control genes and technical repeats are performed to reduce confounding variance. Several strategies have been proposed for normalizing real-time RT PCR data. These range from ensuring that a similar sample size is chosen to using an internal housekeeping or reference gene. These approaches are not mutually exclusive and can be incorporated into a protocol at many stages.

Among all the different method for normalization, normalizing to a reference gene is a simple and popular method for internally controlling error in real-time RT-PCR. This strategy targets RNAs encoded by genes, which have been collectively called housekeeping genes and benefits from the fact that all the steps required to obtain the final PCR measurement are controlled for. The procedure is simplified as both the gene of interest and the reference genes are measured using real-time RT-PCR. Reference genes can also control for different input RNA amounts used in the reverse transcription step however, because this can vary with reverse transcriptase type so it must be validated.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) has been demonstrated to be particularly suitable for the analysis of weakly expressed genes, such as those encoding transcription factors. Additionally, using different rice cultivars, tissues and physiological conditions, they evaluated the expression stability of seven reference genes (Caldana *et al.*, 2007).

2.7.4 Expression studies

Guimil *et al.* (2005) observed conservation of the transcriptional response of rice to colonization by symbionts and pathogens, and a portion of these molecular components are shared between mono and dicotyledonous plants which was confirmed with real-time RT-PCR. McGrath *et al.* (2005) identified transcription factors (TFs) involved in jasmonate (JA) signaling and plant defense.

They screened 1,534 Arabidopsis (*Arabidopsis thaliana*) TFs by real-time quantitative reverse transcription-PCR for their altered transcript at 6 h following either methyl JA treatment or inoculation with the incompatible pathogen *Alternaria brassicicola.* They identified 134 TFs that showed a significant change in expression, including many ethylene response factor (AP2/ERF), MYB, WRKY, and NAC TF genes with unknown functions.

Wen *et al.* (2005) studied certain isolates of nonpathogenic binucleate *Rhizoctonia* spp. (np-BNR) are effective biocontrol agents against seedling root rot and damping- off. Inoculation of bean seed with np-BNR strain 232-CG at sowing reduced disease symptoms in bean (*Phaseolus vulgaris*) seedlings caused by *R. solani*. Molecular analyses of the spatial expression of three defense-associated genes were carried out using real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assays.

Mei *et al.* (2006) studied that exogenous application of JA was able to activate defense gene expression and local induced resistance in rice seedlings against the rice blast fungus (*Magnaporthe grisea*). Furthermore, they characterized a pathogen-inducible rice *OsAOS2* gene (which encodes allene oxide synthase, a key enzyme in the JA biosynthetic pathway) and examined the role of endogenous JA in rice defense response through transgenic manipulation of the JA biosynthesis by using qRT PCR analysis.

Vergne *et al.* (2007) studied rice genes differentially expressed during early stages of *Pi33* / *ACE1* (avirulence related genes) interaction were identified using DNA chip-based differential hybridization and qRT-PCR survey of the expression of known and putative regulators of disease resistance.

Qiu *et al.* (2007) studied that OsWRKY13, as an activator of the SA dependent pathway and a suppressor of JA-dependent pathways, mediates rice resistance by directly or indirectly regulating the expression of a subset of genes acting both upstream and downstream of SA and JA. Furthermore, OsWRKY13 will provide a transgenic tool for engineering wider-spectrum and whole-growth-stage resistance rice in breeding programs.

Lehtonen *et al.* (2008) monitored induction of defense-related responses in the apical portion of potato sprouts using microarray and quantitative polymerase chain reaction techniques at 48 and 120 h postinoculation (hpi) and by challenge-inoculation with *R. solani* in experiments.

Chi *et al.* (2009) studied that the counterdefense mechanism of the fungus against plant-driven ROS. They found that a pathogenicity factor from rice blast fungus, DES1 (Defense Suppressor 1), is involved in overcoming oxidative stress for the counter defense mechanism, suggesting that this gene is required for fungal pathogenicity.

Hao *et al.* (2010) analyzed the inducible expression of defence genes by using quantitative real-time polymerase chain reaction and activities of antioxidant enzymes in the necks, at both the booting stage (BS) and full heading stage (FHS), after inoculation with *Magnaporthe oryzae*. They found that defence genes *PR1b* (pathogenesis-related class 1b), *PBZ1* (probenazole-inducible gene), *PAL* (phenylalanine ammonia-lyase), and *CHS* (chalcone synthase) may play roles in the resistance difference to neck blast between the BS and FHS in rice variety Jiajing3768.

Liu *et al.* (2010) elucidated the molecular mechanisms of phenotypic divergence in oxidative stress response observed between Nipponbare (japonica variety) and 93-11 (indica variety), they conducted a microarray analysis and real-time RT-PCR validation to identify genes differentially expressed in response to methyl viologen (MV) treatment in the two rice cultivars. They identified Core Intersectional Probeset, and further employed multiple enrichment analysis to understand the genetic basis of oxidative stress resistance.

Rawat *et al.* (2010) identified a sequence encoding putative pathogenesis-related protein *OsPR10α* (GenBank accession no. GQ487633) from suppression subtractive hybridization cDNA library of rice cultivar Suraksha, after infestation with the Asian rice gall midge (*Orseolia oryzae*) involving incompatible interaction with hypersensitive reaction (HR). Full-length cloning (RACE) and homology search proved its identity as root-specific *OsPR10α* (*RsOsPR10α*). The expression of *RsOsPR10α* was analysed at different time intervals to understand its role in the resistance mechanism in rice against the pest. Real-time PCR detected significant upregulation of the gene, 40.4 and 23.5-fold at 24 and 120 h after infestation respectively, with the avirulent gall midge biotype 1 (GMB1) in Suraksha.

Sharon *et al.* (2011) demonstrated that the three resistance pathways SAR, ISR, and the phytoalexins production system were induced by colonization with the protective hypovirulent *Rhizoctonia* isolates that protected plants against pathogenic *R. solani*. Changes were also observed in protection levels of *Arabidopsis thaliana* mutants defective in defense-related genes by using qRT-PCR analysis.

Hussain *et al.* (2012) conducted a field experiment to illustrate the different degree and dynamics of microbial community structure and function in the rhizosphere across four growing stages (before plantation and three growth stages) using a combination of biochemical (enzyme assay and microbial biomass carbon) and molecular approaches of qPCR and PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis). Rice plant cultivation promoted higher enzyme activities (invertase and urease), microbial biomass carbon (Cmic), bacterial (16S rRNA) and fungal (ITS rRNA) genes abundances in the rhizosphere compared to unplanted soil.

Kyndt *et al.* (2012) studied the differential expression of genes involved in stress and defence responses in systemic tissue of rice infected with the root knot nematode (RKN) *Meloidogyne graminicola* and the migratory root rot nematode *Hirschmanniella oryzae,* two agronomically important rice pathogens with very different lifestyles. qRT-PCR revealed that all investigated systemic tissues had significantly lower expression of isochorismate synthase, a key enzyme for salicylic acid production involved in basal defence and systemic acquired resistance. The systemic defence response upon migratory nematode infection was remarkably similar to fungal rice blast infection.

Ritesh Kumar *et al.* (2012) carried out ‘functional screening’ of a cDNA library (made from a salt tolerant rice—Pokkali) to identify new ‘‘candidate genes’’ having potential role in salinity tolerance. Based on this screening, they identified a cDNA clone that was allowing yeast cells to grow in the presence of 1.2 M NaCl. Sequencing and BLAST search identified it as mannose-1- phosphate guanyl transferase (*OsMPG1*) gene from rice. Analysis of rice genome sequence database indicated the presence of 3 additional genes for MPG. Out of four, three MPG genes *viz. OsMPG1*, 3 and 4 were able to functionally complement yeast MPG mutant YDL055C. They have carried out detailed transcript profiling of all members of MPG family by qRT-PCR using two contrasting rice genotypes (IR64 and Pokkali) under different abiotic stresses.

MATERIAL AND METHODS

In the present investigation, the laboratory and pot culture experiments were conducted in the Department of biotechnology, College of Agriculture, University of Agricultural Sciences, Dharwad and the methology adopted detailed hereunder.

3.1 Isolation of actinomycetes from soil sample

1. Soil sample was collected from rhizosphere of healthy rice crop from Mugad Rice Research Station (MRRS) is situated at $15^0$15 □ North latitude and 74^0 40□ East longitude at an altitude of 697m above the mean sea level.
2. Weighed 100 gm of soil and suspended in 100 ml of distilled water.
3. Suspension culture of soil was serially diluted up to 10^{-3}.
4. 100 µl of dilution each from 10^{-2} and 10^{-3} dilutions was spread on Starch Casein Agar (SCA) medium (Appendix) containing cycloheximide (100 µg/ml) and streptomycin (50 µg/ml).
5. The plates were incubated at 28^0C for 3 weeks (Shantikumar *et al.* 2006).

3.2 Maintenance of pure cultures

The preservation of actinomycetes was done by following method;

A loopfull of well grown subcultured actinomycetes was picked by using inoculating loop and inoculated in 800 µl of Starch Caesin Broth, kept in shaker at 175 rpm for five days to get some mycelial growth. After five days, 200 µl of glycerol was added to each tube. After proper labelling they were kept in -80^0C.

3.3 Molecular characterization of actinomycete isolates

3.3.1 DNA isolation of actinomycetes

3.3.1.1 From suspension culture

The chromosomal DNA was isolated by versatile quick prep method for genomic DNA of Gram positive bacteria (Pospiech and Neumann, 1995). Actinomycetes suspension (20 ml) grown in LB broth shake culture was centrifuged, and resuspended in 0.4 ml extraction buffer (Appendix). Lysozyme was added to a concentration of 20 mg/ml and incubated at 37^0C for 0.5 to 1h. Then 100µl of 10 per cent SDS was added and incubated at 55^0C for two hours. One third volume of 5M NaCl and one volume of phenol chloroform was added and incubated at room temperature for 30 minutes. The mixture was centrifuged at 10,000 rpm for 10 minutes and aqueous phase was transferred to a new tube. The chromosomal DNA was precipitated by the addition of one volume of iso-propanol with gentle inversion. The DNA was transferred to a new tube, rinsed with 70 per cent alcohol and air dried and dissolved in suitable volume (about 100µl) of 10mM Tris-EDTA.

3.3.1.2 From actinomycetes mycelial mat

The method of DNA isolation described by Kumar *et al*., 2010 was followed. Actinomycetes were incubated aseptically into 250ml flasks each containing 30ml of nutrient broth medium (Appendix) and kept on desk without disturbance for 7 days. The 0.1gm of mycelium was transferred into sterile porcelain dish and crushed with liquid nitrogen. The crushed mycelium was transferred into fresh tube containing 500µl of TE buffer supplemented with lysozyme (20mg/ml). The tube was incubated at 37^0C for 30 minutes. 20µl of 10 per cent SDS (w/v) and 20µl of proteinase K were added into the tube and incubated at 55^0C for 30 minutes. The lysate was cooled down and extracted once with equal volume of phenol: chloroform: isoamyl alcohol (IAA) (25:24:1) at 10,000 rpm for 10 minutes. Again, they were extracted with chloroform and IAA (24:1). The aqueous phase was transferred to a fresh tube; DNA was precipitated with 100 per cent isopropanol and kept at -20^0C for 30 minutes.

The pellet was formed by centrifuging at 10,000 rpm for 10 minutes. The pellet was washed by adding 100µl of 70 per cent ethanol and dissolved in TE buffer. To obtain RNA free DNA, 20µl of RNase solution (20µg/ml) was added and the sample was once again extracted with equal volume of phenol: chloroform: isoamyl alcohol and precipitated as mentioned above. The purity and concentration was checked by using ND-1000 Spectrophotometer.

3.3.1.3 PCR amplification of 16S rDNA region

The oligonucleotide primer for amplification of 16S rDNA sequence was custom synthesized at Sigma chemical Co., U.S.A and supplied as lyophilised product. Details of 16S rDNA primer sequence given as follows:

Primer	Sequence	Amplified fragment size	Reference
16S rDNA	27f 5'AGAGTTTGATCCTGGCTCAG3' 1492r 5'ACGGCTACCTTGTTACGACTT3'	~1.5kb	Lane (1991)

3.3.1.4 Preparation of reaction mixture:

Every time master mixture was prepared afresh to avoid handling errors. The master mix was distributed to tubes. The composition of reaction mixture taken was as follows:

Reagents	Volume (µl)
Taq buffer B	1
dNTP (1 mM)	1
27f primer (5 pm/µl)	0.5
1492r primer (5 pm/µl)	0.5
$MgCl_2$ (1.25 mM)	0.5
Taq DNA Polymerase (3U/ml)	0.3
Template DNA (100 ng)	1
Nuclease free BSA (20 mg/ml)	0.4
100 per cent DMSO	0.4
Sterilized distilled water	4.4
Total	10

3.3.1.5 PCR amplification condition

Eppendorf Master Cycler (5331) was used to run the PCR programme. The PCR amplification protocol employed for amplification was initial denaturation at 95^0C for 5 min, followed by 35 cycles of denaturation at 94^0C for 1 min, annealing at 57.3^0C for 1 min and extension at 72^0C for 1 min 30 s. A final extension was done at 72^0C for 10 min.

3.3.1.6 Separation of amplified product by gel electrophoresis

Products of PCR were separated electrophoretically and EtBr was used for visualization of bands. PCR amplified products from each reaction along with 3 µl of loading dye (Bromophenol blue) (Appendix) was loaded on 0.7 per cent agarose gel. The electrophoresis was carried out using 1X TAE buffer (pH-8.0) (Appendix) at 60V. 1kb DNA ladder from Fermentas was run in a separate lane. The DNA bands on the gels were visualized under UV transilluminator and documented using a gel documentation system.

3.2.2 PCR amplification of ITS region

The Oligonucleotide primer for the amplification of ITS region of 18S rDNA sequence of the fungus *Colletotrichum* sp. was custom synthesized at Sigma Chemical Co., U.S.A and supplied as lyophilized product.

Primer details of ITS region of 18S r DNA sequences

Primer	Sequence	Amplified fragment size	Reference
18S rDNA	ITS1 5'TCCGTAGGTGAACCTGCGG3' ITS4 5'TCCTCCGCTTATTGATATGC3'	600-800 bp	White *et al.*, 1990

3.2.2.1 Preparation of reaction mixture:

Every time the master mixture was prepared afresh to avoid handling errors. The master mix was distributed to tubes. The composition of reaction mixture taken is as follows:

Reagents	Volume (µl)
Taq buffer B	1
dNTP (1 mM)	1
ITS1 primer (5 pm/µl)	0.5
ITS primer (5 pm/µl)	0.5
$MgCl_2$ (1.25 mM)	0.5
Taq DNA polymerase (3 U/ml)	0.3
Template DNA (100 ng)	1
Sterilized distilled water	5.2
Total	10

3.2.2.2 PCR amplification condition

Eppendorf Master Cycler (5331) was used to run the PCR program. The PCR amplification protocol employed for amplification was initial denaturation at 94^0C for 5 min, followed by 35 cycles of denaturation at 94^0C for 5 min, followed by 35 cycles of denaturation at 94^0C for 30 s, annealing at 56^0C for 30 s, and extension at 72^0C for 30 s. A final extension was done at 72^0C for 7 min.

3.2.2.3 Separation of amplified product by gel electrophoresis

Products of PCR were separated electrophoretically and EtBr was used for visualization of bands. PCR amplified products from each reaction along with 3 µl of loading dye (Bromophenol blue) (Appendix) was loaded on 0.7 per cent agarose gel. The electrophoresis was carried out using 1X TAE buffer (pH-8.0) (Appendix) at 60V. *Hind*III double digest ladder from Fermentas was run in a separate lane. The DNA bands on the gels were visualized under UV transilluminator and documented using a gel documentation system.

3.3.2 Rapid identification of filamentous actinomycetes at the genus level

To identify the isolates at genus level, a rapid identification method was followed based upon amplified rDNA restriction analysis (ARDRA) of 16S rDNA. PCR amplification was done by using universal 16S rDNA primers. PCR amplified DNA was initially subjected to digestion with Sau3AI. Samples were electrophoresed on 1.5 per cent agarose gel and subsequent restriction based identification was done as described by Cook *et al.* (2003).

3.4 Microorganism used in study

During this study, bioagents isolated from different places were used. The test pathogen, *R. solani* isolated from three different rice growing places of Karnataka was used.

Table 1: List of Biocontrol agents tested against *R. solani*

Sl. No.	***Trichoderma spp.***	**Location**
1	*T. virens* (1002)	Unknown
2	*T. virens* (1006)	Dharwad
3	*T. virens* (1010)	Gujarat
4	*T. koningii* (1016)	Dharwad
5	*T. harzianum* (1032)	Unknown
6	*T. harzianum* (1034)	Kalkeri
7	*T. harzianum* (1036)	Kalkeri
8	*T. harzianum* (1040)	Kuluvane leaf litter
9	*T. harzianum* (1041)	Neernalli Farm
10	*T. harzianum* (1042)	Neernalli Farm
11	*T. harzianum* (1043)	Neernalli Farm
12	*T. viride* (1044)	Janmani runoff soil
13	*T. harzianum* (1046)	Malagi
Sl. No.	Fluorescent pseudomonads	Location
1	Fluorescent pseudomonad (15)	Pomegranate rhizosphere of Koppal district
2	Fluorescent pseudomonad (1)	
3	Fluorescent pseudomonad (30)	
4	Fluorescent pseudomonad (19)	
5	Fluorescent pseudomonad (25)	
6	Fluorescent pseudomonad (22)	
7	Fluorescent pseudomonad (12)	
8	Fluorescent pseudomonad (6)	
9	Fluorescent pseudomonad (20)	
10	Fluorescent pseudomonad (Soy6)	Dept. of microbiology, UAS Dharwad
11	Fluorescent pseudomonad (Soy2)	Dept. of microbiology,UAS Dharwad
12	Fluorescent pseudomonad (Wht1)	Dept. of microbiology, UAS Dharwad
13	Fluorescent pseudomonad (Grnrt1)	Dept. of microbiology,UAS Dharwad
14	Fluorescent pseudomonad (Safrrt1)	Dept. of microbiology,UAS Dharwad
15	Fluorescent pseudomonad (IOF3)	Institute of organic farming,UAS Dharwad

Table 1: Contd…..

Sl. No.	**Pink pigmented facultative methyllotrophs**	**Location**
1	H5	Koppal
2	75L	Bheemarayanagudi
3	38U	Raichur
4	42U	Raichur
5	23A	Gangavati
6	20A	Hampi
7	27U	Gangavati
8	71L	Gulberga
9	10L	Gadag
10	26U	Gangavati
11	32	Raichur
Sl. No.	*Actinomycetes spp.*	Location
1	IABT-A1	Mugad
2	IABT-A2	
3	IABT-A3	
4	IABT-A4	
5	IABT-A5	
6	IABT-A6	
7	IABT-A7	
8	IABT-A8	
9	IABT-A9	
10	IABT-A10	
11	IABT-A11	

Fluorescent pseudomonad, Methalobacterium strains and isolates of *Trichoderma* were obtained from and Department of Microbiology and Department of Biotechnology, UAS, Dharwad respectively. Actinomycete isolates were isolated from soil samples collected from healthy rice crop rhizosphere of rice research station (Mugad), Dharwad which are enlisted in Table 1.

3.5 Preparation of culture media

Total 50 isolates of bioagents were used in this study. Thirteen isolates of *Trichoderma*, fifteen isolates of Fluorescent pseudomonad*,* eleven isolates of actinomycete and eleven pink-pigmented facultative methylotrophs were grown on Potato Dextrose agar, King's B agar media, Starch casein Agar medium and Ammonium mineral salt agar medium (supplemented with 0.5 per cent v/v methanol) respectively. The culture media were prepared and pH was adjusted at 6.8 and sterilized by autoclaving at 15 psi for 20 minutes. The media composition is mentioned in Appendix .

3.6 Inoculation of pathogen

3.6.1 Plant materials

Rice variety Samba Mahsuri (BPT – 5204), which is susceptible to sheath blight was used in this study. Rice plants were grown in pots in a greenhouse (28℃ day, 23℃ night, 14 h of light) or under field conditions. Rice plants at tillering stage (4-5 week-old plants) were used for inoculation with *R. solani*.

3.6.2 Fungal inoculum

The two fungal isolates of *R. solani* (Mandya HRL and VC Farm Mandya) used in this study. To compare the effects of different types of inoculum on the severity of sheath blight, three inoculums types were prepared and used including (i) agar block (diameter 0.5 cm) (ii) liquid cultured mycelia ball (diameter 0.5 cm) and (iii) soil treatment. *R. solani* mycelia agar plug was inoculated on potato dextrose agar (PDA) and grown at room temperature (28℃). Agar blocks (0.5 cm squares) were cut and prepared from the outer edge of 3 days old culture was used. In second method, liquid cultures were prepared by inoculating fresh mycelia of pathogen in 200 ml of potato dextrose broth (PDB) taken in 250 ml Erlenmeyer flask and incubated at 130 to 140 rpm on shaker in dark for 7 to 10 days. Liquid cultured mycelia were harvested and cut into small mycelial balls (approximately 0.5 cm in diameter). In third method, Mass inoculum of isolate (Mandya HRL and VC Farm Mandya) was grown in 250 ml flasks containing 100 g of sorghum grains and sand (2:1) plus 120 ml double distilled / deionized water and sterilized for 15 min at 121^{o}C temperature and 15 psi pressure for three-four times (Balali *et al.*, 1995). Each flask was inoculated with five-six agar plugs of 5 mm diameter of the fungus taken from the margins of one week old culture of isolate (Mandya HRL and VC Farm Mandya) grown on PDA medium. Flasks were incubated at 25 ^{o}C for 18 days and further used for inoculation with *R. solani* to rice plant.

3.6.3 Standardization of pathogen inoculation method

Different inoculation methods were tried to standardise the best inoculation method for maximum disease induction in less time. Rice plants at tillering stage were inoculated with *R. solani* (Mandya HRL) by placing a mycelial ball beneath the leaf sheath. The inoculated sheath was covered immediately with aluminium foil. The aluminium foil was removed after 10-15 days, on appearance of typical lesions and covered with polythene bags for 2 weeks to allow for disease development. Plants were grown at 28℃ in the greenhouse. Agar plug of 4 days old cultures was inoculated on sheath and covered with wet cotton. Lesion length on the sheath of the inoculated plants was measured after 15 days of inoculation. Mass multiplied inoculum was mixed with soil and pots were covered with plastic for seven days in third treatment. The experiment was replicated. The results were verified by repeating the experiment two times and re - isolating the *R. solani* from diseased leaves. The part of leaf showing typical symptoms of disease was washed under running tap water and cut into small bits (2-3 mm). These bits were surface sterilized with Sodium hypochlorite (10%) solution for 1min. The bits were washed thoroughly in sterile distilled water for thrice to remove traces of sodium hypochlorite and transferred aseptically to sterilized potato dextrose agar (PDA) plates and were incubated at 27 ± 10^{0}C for three-four days to obtain fungal growth (Rangaswami, 1972).

3.7 Screening of biocontrol agents against *R. solani* under in vitro condition

3.7.1 *Trichoderma* Isolates

The antimicrobial activity was studied by dual culture method. Pure *Trichoderma* isolates was spot inoculated on PDA medium having pathogen and incubated at 28^0C for 3-4 days. (Amin *et al.*, 2010).

In each treatment percent inhibition was calculated by using formula as, I = C-T/C×100 where, I-inhibition of mycelial growth (%), C-growth of pathogen in control (mm), T- growth of pathogen in treatment (mm) (Vincent, 1991).

3.7.2 *Rhizobacterial* strains

3.7.2.1 Fluorescent pseudomonad isolates were screened by streaking on either side of the plate containing King's B medium and agar plug of *R. solani* was placed at centre of streaked culture plate after 48 hr of bacterial growth (Kazempour, 2004).

3.7.2.2 Pink-pigmented facultative methylotrophs were screened by streaking on either side of the plate containing PDA medium and agar plug of *R. solani* was placed at centre of streaked culture Plate after 72 hr of bacterial growth. (Poorniammal *et al.*, 2009).

In each treatment percent inhibition was calculated by using formula as, I=C-T/C×100 where, I-inhibition of mycelial growth (%), C-growth of pathogen in control (mm), T- growth of pathogen in treatment (mm) (Vincent, 1991).

3.7.3 Actinomycetes isolates

The bioassay method followed essentially according to the protocol given by Soares *et al.*, 2006. Actinomycetes were streaked in the middle of the plate containing Soybean-Casein Digest Agar Medium (SCBA) (Basavaraj *et al.*, 2010) (Appendix I).Agar plugs of pathogen were placed on either sides of streaked culture after four days of actinomycetes inoculation. The growth of pathogen was noted periodically.

The percent inhibition was calculated by using following formula

Inhibition (%) = [(growth radius in untreated control- growth radius in treatment) ×100]/ growth radius in untreated control (Taechowisan *et al.,* 2005).

Inhibition zone was measured for each of the isolates of *Trichoderma*, actinomycetes and strains of pseudomonas, PPFM which was showing good inhibition were subjected to statistical analysis.

3.7.4 Statistical Analysis:

The experiment design was Completely Randomised Design and replicated thrice. The results were statistically analysed by following Duncan Multiple Range Test (DMRT).

3.8 Mass multiplication of potent isolates

3.8.1 *Trichoderma* isolates

It was grown in 250 ml flasks containing 100 g of sorghum grains and sand (2:1) plus 120 ml double distilled / deionized water and autoclaved for 15 min at 121°C temperature or 15 PSI for three-four times (Balali *et al.*, 1995). Each flask was inoculated with five-six agar plugs of 5 mm diameter of the fungus taken from the margins of a 1 week old culture of isolate grown on PDA medium and flasks were incubated at 25°C for 20 days. At the time of application, spore load of 10^6 cfu/g was confirmed.

3.8.2 Fluorescent pseudomonad strains

A loopful of different PGPR strains was inoculated to nutrient broth separately and incubated in a rotary shaker at 150 rpm for 48 h at room temperature (28±2ºC). After 48 h of incubation, the broth containing 9×10^8 cfu/ml was used for the preparation of talc based formulation.

To 400 ml of bacterial suspension, one kg of the purified talc powder (sterilized at 105ºC for 12h) 15 g calcium carbonate (to adjust the pH to neutral) and 10 g of carboxy methyl cellulose (CMC) as an adhesive were mixed under aseptic conditions following the method described by Vidhyasekaran and Muthamilan (1995).

The product was shade dried to reduce the moisture content below 20 per cent and then packed in polythene bags and sealed. At the time of application, the population of the bacteria in talc formulation was checked to 2.5 to 3 × 10^8 cfu/g.

3.8.3 Actinomycetes isolates:

A suspension of isolates (5 ml; 10^8 cfu/ml) was inoculated into 250 ml flasks containing 50 ml sterile nutrient medium. After incubation for 48 h at 28 ± 2^0C at 160 rpm, the cell mass of isolates resuspended twice in sterile distilled water. Soil comprising a mixture of sand and clay (4:1, v/v), was air-dried and sieved before use. The delivery medium consisted of 5 per cent (w/w) roughly ground sorghum seeds in soil, 500 g delivery medium was autoclaved at 121^0C for 30 min in 1L Erlenmeyer flasks. Sterile distilled water was added to the formulation to adjust the moisture content to 40 per cent (w/w). After mixing thoroughly, 50 ml inoculum of isolate was added and incubated at 28 ± 2^0C for 10 days. Two 1 g samples were taken from three randomly selected flasks and serial dilutions were made to determine the average concentration of viable cells of isolates in the delivery medium (Getha *et al.*, 2005).

3.9 Evaluation of bioagents against *R. solani* under glasshouse condition

The pot experiment was conducted under glasshouse conditions.

3.9.1 Soil

The black sandy loamy soil was collected from the Main Agricultural Research Station, Dharwad. The soil was autoclaved.

3.9.2 Potting

The plastic pots having 4 kg capacity were filled with the above soil and FYM mixtures. Before planting, each pot received 2.4, 1.2 and 1.2 g of N, P and K respectively as per the package of practices (100: 50: 50 npk/ha) for rice in the form of urea, single super phosphate and muriate of potash.

3.9.3 Seeds

The seeds of susceptible rice variety, BPT-5204 obtained from the Department of Molecular biology and Biotechnology, University of Agricultural Sciences, Dharwad were used in the study. Rice seeds were surface sterilized by immersing in 1.5 per cent sodium hypochlorite solution for five minutes and subsequently rinsed in sterile distilled water for two to three times to get rid of traces of sodium hypochlorite. Finally, the seeds were air dried and sown in pots.

3.9.4 Treatments of pathogen

3.9.4.1 Soil infestation

Mass multiplied inoculum of pathogen *R. solani* mixed with soil @ 4 per cent per kg soil and covered pots with plastic for one day.

3.9.4.2 Sheath application

Agar plug of 4 days old culture was inoculated on sheath of 4 week old plants and covered with wet cotton which was properly bound with aluminium foil to maintain moisture.

3.9.5 Treatments of potent bioagents

The experiment was conducted in pot culture under greenhouse condition with 7 treatments and 3 replications of different potent bioagent following the Completely Randomized Block Design (CRBD).

In this pot study, first four treatments of bioagents was conducted in pathogen (*R. solani* isolate Mandya HRL) infested soil before one day of sowing.

Sl. No.	Treatment	Treatment detail
1	Seed treatment (T1)	Seed treatment of bioagent
2	Soil application (T2)	Soil application of bioagent
3	Foliar spraying (T3)	Spraying of bioagent on leaves
4	Combination (T4)	Seed treatment + Soil application + Foliar spraying of bioagent
5	Only pathogen (T5)	Pathogen inoculation to seed, soil, sheath
6	Only bioagent (T6)	Only bioagent
7	Untreated control (T7)	no pathogen: no bioagent: Normal plant

3.9.5.1 Seed bacterization (Weller and Cook 1983)

Rice seeds were bacterized, following the method given by Weller and Cook (1983) along with some modification for rice seeds. The seeds were treated with PGPR culture having a population of 5×10^7 cfu/ml in suspension culture. It was treated @ 1g of seed per 10 ml suspension along with 0.2 per cent carboxy methyl cellulose. Seeds were air dried to avoid clumping. After air drying, the treated seeds were sown in pots.

3.9.5.2 Soil application method

The talc based culture was properly and equally mixed with autoclaved soil @ 2 per cent per kg soil of bioagent.

3.9.5.3 Foliar spraying method (Kumar *et al.*, 2003)

Two per cent culture suspension having spore load of 10^6 cfu/ml was sprayed at three different intervals *i.e.*, 15, 30 and 45 days after sowing (DAS).

3.9.5.4 Combination of all the three methods

A combination of seed treatment, soil application and foliar spraying method was followed.

3.9.5.5 Only pathogen

Seeds were treated with only pathogen and sown in *R. solani* sick soil and also sheath inoculation of *R. solani* method was followed.

1.9.5.6 Only bioagent

Seeds were treated with only bioagent and sown in autoclaved soil then after 15 days sowing, same culture of bioagent was applied in a soil and 30 days after sowing foliar spraying method was followed.

3.9.5.7 Untreated Control

The rice seedlings were raised without any inoculations and maintained as absolute control.

3.10 Observations recorded

Observations were recorded for five plants in each treatment in each replication.

3.10.1 Disease parameters

3.10.1.1 Lesion length

The pathogen infected sheath and causeed lesions which increased in size further leading to drying of leaves and finally death of the plants.

The lesion length was measured at 40 and 60 days after sowing (DAS).

3.10.1.2 Number of dried leaves

The pathogen infected leaves which finally dried and number of dried leaves per plant was recorded at 40 DAS.

3.10.2 Growth parameters

3.10.2.1 Plant height

The plant height was measured from the base of the plant upto the tip of the fully opened top leaf at 40 and 60 days after sowing.

3.10.2.2 Number of tillers

Number of tillers for each plant samples was counted at 40 DAS.

3.10.2.3 Root length

The Root length was measured from the base of the plant upto the tip of root after uprooting the plant at 75 days after sowing

3.10.2.4 Total biomass content

The uprooted plants were oven dried at 60^0C to a constant weight. The dry biomass was then recorded and expressed in grams per plant.

3.10.4 Statistical analysis

The data obtained in the present investigations for various parameters were subjected to ANOVA for a completely randomized design for *in vitro* studies and randomized complete block design for *in vivo* studies by using M-STAT C programme (Gomez and Gomez, 1984).

3.11 Molecular interaction

The potential bioagent against *R. solani* was identified based on its disease control ability and plant growth promoting characters. This isolate was used as an ideal bioagent for further understanding of gene expression changes happening in response to infection by *R. solani* in rice-bioagent- pathogen interaction.

3.12 Total RNA isolation and handling

3.12.1 General precautions

Before starting RNA isolation, certain precautions were strictly followed to avoid contamination and degradation; so as to obtain intact and high quality RNA. All the glassware, mortar and pestle, microtips and microcentrifuge tubes were treated with autoclaved DEPC treated water and then autoclaved. Solutions like 75 percent ethanol and MOPS buffer were prepared with autoclaved DEPC treated water. Electrophoresis unit was first wiped with RNAse ZAP and then washed with DEPC treated water. Gloves were worn throughout the experiment.

3.12.2 Sample collection

For RNA isolation rice leaves from different treatment of actinomycete isolates (IABT-A7) were collected. Hundred gram of rice leaves from seed treatment, combine treatment, only bioagent treatment, treated control (pathogen) and untreated control were taken at different intervals of 24 h, 48h, 72h and 120 h after inoculation of pathogen to sheath.

3.12.3 RNA isolation

1. The total RNA was extracted from different treated plant tissues and control tissues using TRIZOL reagent (Invitrogen, San Diego USA).
2. The tissue sample of 100 g was weighed and ground quickly using a DEPC treated pre-chilled pestle and mortar until the sample turned into very fine powder. The sample was never allowed to thaw.
3. The ground tissue was transferred to a pre-chilled 50 ml conical tube and TRIZOL reagent (1 ml of TRIZOL/100 mg of tissues) was added.
4. The contents were mixed well by vortexing and 1 ml of liquid phase was transferred into RNase-free 1.5 ml tubes and incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes.
5. After incubation, 200 µl of chloroform per 1ml of TRIZOL was added and vortexed for 20 sec followed by incubation for 10min at room temperature.

6. The samples were centrifuged at 13,200 rpm for 10 min at 4^0C. After centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase.
7. The aqueous phase containing RNA was carefully transferred to a new RNase-free 1.5 ml tube without disturbing the other phases.
8. The tube was placed on ice as soon as possible. An equal volume (~ 0.6 ml) of isopropyl alcohol (isopropanol) was added and mixed gently by inverting (2-3 times).
9. The samples were incubated on ice for 30 min for precipitating the RNA and centrifuged at 13,200 rpm for 20 min at 4^0 C. A very small gel like pellet was visible on the side and the bottom of the tube.
10. The supernatant was discarded and the tubes were let to stand upside down on Kimwipes (Kim Tech Science, Canada). The pellet was washed with 1 ml of 75 per cent ethanol by gently vortexing and centrifuging.
11. The liquid was decanted and the inside of the tube was wiped with a clean kimwipe to dry the pellet without touching it.
12. The pellet was resuspended in 500 µl of RNase-free water by flicking the tube. This was incubated on ice for at least an hour and pipetted occasionally for dissolving and centrifuged at 13,200 rpm for 20 min at 4^0C.
13. Sodium acetate precipitation was done by transferring to a new RNase-free 1.5 ml tubes. Precipitation was achieved by adding 10 per cent volume of 3M sodium acetate and equal volume of isopropanol.
14. The contents were incubated on ice for overnight at -80^0C and next day, centrifuged at 13,200 rpm for 20 min at 4^0C.
15. The supernatant was decanted and the pellet was washed once again with 1 ml of 75 per cent ethanol and the final pellet was resuspended in 50 µl of RNase-free water. This was stored at-80^0C till further use.

3.13 Quality and quantity analysis of total RNA

3.13.1 Quality analysis by agarose gel electrophoresis:

The overall quality of the RNA preparation was assessed by fractionating the samples over formaldehyde denaturing agarose gel (Appendix) following the method as described by Sambrook *et al.* (1989).

3.13.1.1 Agarose gel containing 2.2 M formaldehyde

1. 100 ml of a 1.5 per cent agarose gel containing 2.2 M formaldehyde was prepared by adding 1.5 g of agarose to 72 ml of sterile H_2O.
2. The agarose was dissolved by boiling in a microwave oven. The solution was cooled to 55^0C and 10 ml of 10x MOPS electrophoresis buffer and 18 ml of deionized formaldehyde was added. In a chemical fume hood, an agarose gel was casting with slots formed by a 3mm comb with at least four more teeth than the number of RNA samples under test. These extra lanes are used for RNA size markers and running dyes. The gel was allowed to set for at least 1 hour at room temperature. As soon as the agarose has set, the gel was covered with Saran Wrap until the samples were ready to be loaded.

3.13.1.2 Special Equipment

Equipment for horizontal electrophoresis

A particular electrophoresis apparatus should be reserved specifically for RNA analysis. Electrophoresis tanks and combs used for electrophoresis of RNA was cleaned with detergent solution, rinsed in H_2O, dried with ethanol, and then filled with a solution of 3 per cent H_2O. After 10 minutes at room temperature, the electrophoresis tanks and combs were rinsed thoroughly with H_2O treated with 0.1 per cent DEPC.

Because the pH of the electrophoresis buffer changes during the run, the electrophoresis tank was set up, so that the buffer circulates continuously from one chamber to the other via a peristaltic pump. Alternatively, transfer the buffer manually every hour or so from one buffer chamber to the other.

Water bath was preset to 55°C

Method

1. The denaturation reaction was set. In sterile microfuge tubes mix was as follows:

RNA (up to 20 µg)	2.0 µl
10x MOPS electrophoresis buffer	2.0 µl
Formaldehyde	4.0 µl
Formamide	10.0 µl
Ethidium bromide (200 µg/ml)	1.0 µl

As much as 20µg of RNA may be analyzed in each lane of the gel.

2. The tops of the microfuge tubes were closed and the RNA solution was incubated for 60 minutes at 55^0C. The samples were chilled for 10 minutes in ice water, and then sample was centrifuged for 5 seconds to deposit all of the fluid in the bottom of the microfuge tubes.
3. 2 µl of 10x formaldehyde gel-loading buffer was added to each sample and the tubes were again kept back to an ice bucket.
4. The agarose/formaldehyde gel was installed in a horizontal electrophoresis box. Then added sufficient 1x MOPS electrophoresis buffer to cover the gel to a depth of ~1 mm. The gel was run for 5 minutes at 5 V/ cm and then loaded the RNA samples into the wells of the gel, leaving the two outermost lanes on each side of the gel empty.
5. The gel was run which was submerged in 1x MOPS electrophoresis buffer at 4-5 V/ cm until the bromophenol blue had migrated ~8 cm (4-5hours).
6. The RNA was visualized by placing the gel on a piece of Saran Wrap on a UV transilluminator.

3.13.2 Spectrophotometric analysis of RNA

The quantity and quality of RNA was estimated using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). Before taking sample readings, the instrument was set to zero by taking 1µl autoclaved distilled water as blank. One microlitre from each sample was quantified and was measured in ng/µl. The absorbance of nucleic acid samples were measured at a wavelength of 260 nm and 280 nm and OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios were recorded to assess the purity of RNA. A ratio of 1.8 to 2.0 for OD_{260}/OD_{280} and above 1.0 for OD_{260}/OD_{230} indicated good quality RNA.

The total RNA was stored at -80^0C for future use.

3.14 Purification of total RNA

The total RNA was treated to remove the DNA contamination using TURBO DNA-free ™ kit (Cat# AM 1907 Ambion, USA) as per the instructions in the manual supplied by the company. In order to eliminate the genomic DNA, 10 µg of total RNA was digested with RNase-free DNaseI and finally the total RNA was precipitated into a desired volume of water keeping subsequent application in view. Further, resulting quantity and quality of RNA samples were checked by using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA).

3.15 Preparation of cDNA

The desired quantity of DNA free total RNA was converted to single stranded cDNA by using HighCapacity™ cDNA Reverse Transcription kit (Cat#4374966, Ambion, USA) following the instructions in the manual provided by the company.

All the reagents were thawed and stored on ice in RNase and DNase free work environment. Upon thawing, the reagents were mixed thoroughly and spun down before pipetting. About 2µg of total RNA in a 20 µl reaction was quantitatively converted to single-stranded cDNA using thermal condition mention below;

SI. No.	Components	Volume/reaction (µl)
1	10XRT buffer	2.00
2	25X dNTP (100 mM)	0.80
3	10X RT random primers	2.00
4	MultiScribe^{TM} reverse transcriptase	1.00
5	RNase inhibitor	1.00
6	Nuclease free water	3.20
7	Total RNA (2 µg)	10.00
	Total volume	20.00

3.15.1 Thermal condition for single stranded cDNA conversion

	Step 1	Step 2	Step 3	Step 4
Temperature	25^{0}C	37^{0}C	85^{0}C	4^{0}C
Time	10 min	120 min	5 sec	hold

3.16 Standardization of real-time conditions

3.16.1 Standardization of primer temperature

The selected defence gene primers at 5 pM concentration were standardized for annealing temperature by gradient PCR for each set of primers. The temperature which gave expected size amplicons of good intensity was selected for rest of the procedure. Primer sequences were used as mentioned follows, (Kyndt, 2012).

Target gene	Forward primer	Reverse primer	Mainly involved in	references
OsAOS2	CAATACGTGTACTGGTCGAATGG	AAGGTGTCGTACCGGAGGAA	JA biosynthesis	Agrawal et al. (2004)
OsJMT1	CACGGTCAGTCCAAAGATGA	CTCAACCGTTTTGGCAAACT	MeJA biosynthesis	Seo et al. (2001)
OsJAMYB	GAGGACCAGAGTGCAAAAGC	CATGGCATCCTTGAACCTCT	JA response	Lee et al. (2001)
OsICS1	TGTCCCCACAAAGGCATCCTGG	TGGCCCTCAACCTTTAAACATGCC	SA biosynthesis	Lee et al. (1995)
OsNPR1	CACGCCTAAGCCTCGGATTA	TCAGTGAGCAGCATCCTGACT	SA response	Yuan et al. (2007)
OsPAL1	TGTGCGTGCTTCTGCTGCTG	AGGGTGTTGATGCGCACGAG	SA biosynthesis	Lee et al. (1995)
OsEIN2	TAGGGGGACTTTGACCATTG	TGGAAGGGACCAGAAGTGTT	ET signalling	Jun et al. (2004)
OsPR1b	GGCAACTTCGTCGGACAGA	CCGTGGACCTGTTTACATTTT	SA response	Agrawal et al. (2000)
OsERF1	AAGGGTCATAATTCGCGTCA	TCCACACCACAAGACATCGT	ET response	Hu et al. (2008)
OsACO7	GGACTACTACCAGGGCACCA	GATTAGCGCACGCGATTTTA	ET biosynthesis	Iwai et al. (2006)
OsACS1	GATGGTCTCGGATGATCACA	GTCGGGGGAAAACTGAAAAT	ET biosynthesis	Iwai et al. (2006)

3.16.2 Preparation of reaction mixture

Every time master mixture was prepared afresh to avoid handling errors. The master mix was distributed to tubes. The composition of reaction mixture taken was as follows:

Reagents	Volume/tube (µl)
Sterile distilled water	5.75
Taq assay buffer with $MgCl_2$ (10X)	1.0
dNTPs (1mM)	1.0
Forward primer (5 pM)	0.5
Reverse primer (5 pM)	0.5
Taq DNA polymerase (3U/ µl)	0.25
Template cDNA (100 ng)	1.0
Total	10.0

3.16.3 PCR amplification condition

PCR amplification conditions for selected defence gene primers are given below.

Step	Temperature (^{0}C)	Duration	No. of cycles
Initial denaturation	94	5 min	1
Denaturation	94	30 sec	35
Annealing (For individual pairs of primers)		30 sec	
Os AOS2	50.4		
Os JMT1	50.4		
Os JAMYB	55.2		
Os ACO7	59.1		
Os ACS1	60.5		
Os EIN2	55.4		
Os NPR1	63.0		
Os PAL1	59.1		
Os ERF1	60.0		
Os ICS1	59.1		
Os PR1b	59.1		
Initial extension	72	30 sec	
Final extension	72	10 min	1
Hold	4	-	-

3.16.4 Separation of the amplicons by agarose gel electrophoresis

Ten µl of the reaction mix from each tube along with 3 µl of 6X loading dye (Appendix) were loaded onto 2.5 per cent agarose gel in 1X TAE (Appendix) of pH 8.0 and 100 bp DNA ladder (Bangalore Genei, Bangalore) was used as DNA molecular weight marker. The choice of the marker and percentage of agarose gel were based on the expected size of the amplicons. Electrophoresis was done at 60V. The DNA bands in the gel were visualized on a UV-transilluminator and documented using a gel documentation system (G BOX from Syngene, Beacon House Nuffield Road Cambridge CB4 1TF UK).

3.16.5 qRT-PCR reaction

The reaction mixture of 10 µl containing 5.0ng cDNA, 5.0 pmol of each gene-specific primer and 5 µl of 2X SYBR® Green reagent (Cat. #4368706, Ambion, USA) were used in each reaction. Individual components of reaction mixture were standardized for 10 µl reaction volume. The master mix of different components of real-time PCR was prepared fresh to avoid handling errors.

3.16.6 Internal control

Transcripts of stably expressed genes (reference genes) are generally used for data normalization. The genes such as *18S rRNA, EF-la, beta-actin, beta-tubulin,* and *ubiquitin* (*UBQ)* have served as good reference genes in rice and Arabidopsis (Caldana *et al*., 2007 and Czechowski *et al*., 2004). We initially selected three genes, *viz*, *actin, 18S-rRNA*, and *Elongation factor-la* which are common housekeeping genes in plants. The gene expression stability measure (M) was estimated in a set of different cDNA samples originating from absolute control, pathogen inoculated, only bioagent treated and pathogen-bioagent inoculated tissues using NormFinder software which works on model-based variance estimation approach and which were set as default programme in Eppendorf Mastercycler® ep *realplex* instrument (Claus *et al*., 2004).

The difference in gene expression level of two ideal control genes should be the same in all experimental conditions (tissues, treatment) compared: the lower the M value of a gene, the more stably it is expressed. The M value has a suggested limit of $M<1.5$. In the present experiment, the affect of biotic stress was tested on the expression stability of the selected reference genes.

3.17 Real-time PCR condition

An Eppendorf Mastercycler® ep *realplex* instrument (Eppendorf Pvt. Ltd., Germany) was used for all amplification are given as follows.

Real –time PCR amplification conditions

Stage	Step	Temperature (^{0}C)	Duration (min. and sec.)	No. of cycles
I.	Initial denaturation	95	10 min.	1
II.	Denaturation	95	30 sec.	40
III.	Annealing	60	30 sec.	
IV.	Extension	72	30 sec.	
V.	Melting curve	95	10 min.	1

3.18 Data Analysis for gene expression

3.18.1 Relative gene expression quantification

Absolute quantification and relative quantification are methods to analyze data from real-time PCR experiments. Relating the PCR signal to a standard curve absolute quantification method determines the copy number of a gene. The relative quantification relates the PCR signal of the target transcript in a treatment group to that of other samples such as an untreated control and determines relative fold change in gene expression. In this experiment, the relative quantification approach was used to measure the expression level of 11 defense genes in leaf tissues of different treatment of actinomycete isolate (IABT-A7) as seed treatment, combine treatment, only bioagent treatment, only pathogen (*R. solani*) treatment and absolute control. The mathematical model delta-delta Ct method (Livak and Schmttgen, 2001) was used to determine relative expression ratio (fold change).

The statistical data analysis in gene quantitation is performed on the basis of classical standard parametric test such as correlation coefficient, standard deviation and t-test. The technical precision or reproducibility of real-time PCR measurement was assessed by performing replicate measurements in separate PCR runs (technical replicate and biological replicate). Correlation coefficient was calculated between two technical replicate (technical replicate: same pool of cDNA and biological replicate: two different pools of cDNA obtained independently from different batch of total RNA) to determine the reproducibility of the experiment was done by LinRegPCR software. The standard deviation of ΔCt was calculated from the standard deviation of the target and reference values using the formula $S = (S1^2 + S2^2)^{1/2}$ (S1 = standard deviation of target gene and S2 = standard deviation of reference gene). Coefficient of variance was estimated from replicate Ct values was carried through to the final calculation of relative quantities using standard propagation of error methods. Microsoft Excel programme was used to calculate above statistical parameters.

EXPERIMENTAL RESULTS

The present investigation was undertaken to identify potential biocontrol isolates of *Trichoderma*, *Rhizobacteria* and Actinomycets against rice sheath blight pathogen, *Rhizoctonia solani* and to understand the expression of different defense genes in host during host-pathogen-bioagent interaction. The results obtained from this investigation are detailed below.

4.1 Selective isolation of actinomycetes

Actinomycetes colonies were isolated from the soil sample collected from rhizosphere of healthy rice crop from Mugad Rice Research Station. The actinomycetes were isolated on starch casein agar by spread plate technique as explained in materials and method. The colonies appeared after 20-30 days of incubation and their morphological characteristics were recorded.

Observation on colony morphology, pigmentation and texture are mentioned in Table 2 and shown in Plate 1. Few isolates produced pigmentation like yellow colour (IABT-A3, IABT-A10), red colour (IABT-A1, IABT-A11).Some of them showed texture like Powdery (IABT-A5, IABT-A6, IABT-A7), Solid substratum (IABT-A4, IABT-A8, IABT-A9) and compact (IABT-A2). They were putatively identified as actinobacteria and selected for further purification.

4.2 Molecular characterization of actinomycetes isolates

4.2.1 Isolation of DNA from actinomycetes

Actinomycetes DNA was isolated from two different methods *viz.*, suspension culture and mycelial mat. Among them DNA isolated from suspension culture was found to yield good quality and quantity DNA.

4.2.2 PCR based identification of actinomycetes

The isolates were subjected to 16S rDNA test of prokaryotes (16S rDNA region) and ITS test of eukaryotes (18S rDNA region). PCR amplification was obsereved in all eleven isolates in 16S rDNA test with amplicon size of approximately 1.5kb (Plate 2) but no amplification was seen in ITS test which proved that all isolated cultures were belonging to prokaryotes at genetic level.

4.2.3 Identification of actinomycete isolates at genus level

A rapid identification method was followed to identify actinobacteria at genus level using genus specific 16S rDNA gene restriction fragment patterns that is Amplified rDNA Restriction Analysis (ARDRA) test. ARDRA confirmed that three isolates belonged to *Actinopolymorpha spp.* and remaining eight isolates were grouped under *Streptomyces spp.* (Table 3a and 3b and Plate 3). Isolate IABT-A2, IABT-A6 and IABT-A7 belonged to *Actinopolymorpha spp.* and isolate IABT-A1, IABT-A3, IABT-A4, IABT-A5, IABT-A8, IABT-A9, IABT-A10 and IABT-A11 belonged to *Streptomyces spp.*

4.3 Maintenance of pure culture of biocontrol agents:

The preservation of pure culture of bioagents was done as explained in materials and methods.

4.4 Inoculation and isolation of pathogen

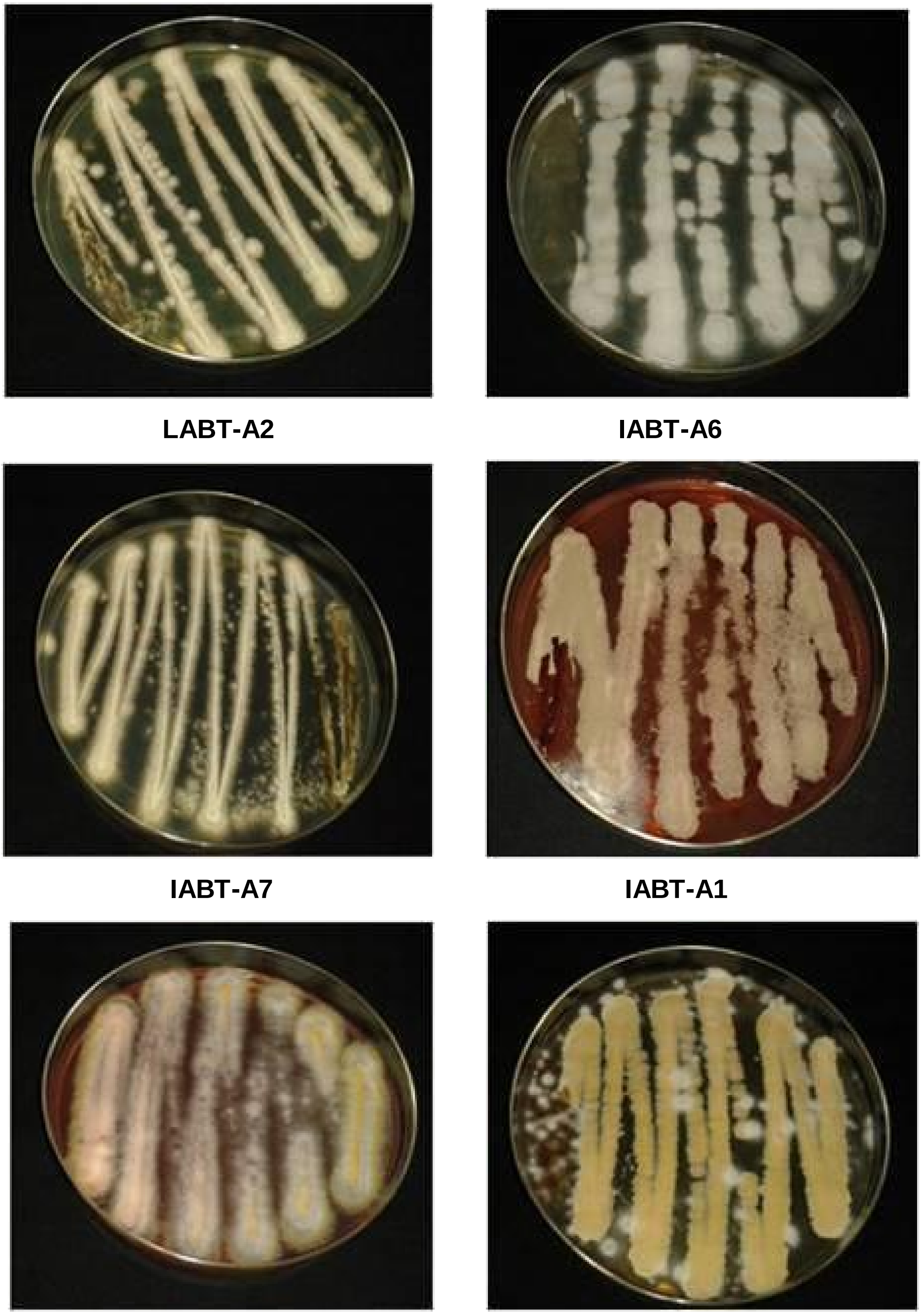
The isolates of *R. solani* were isolated from infected rice sheath by tissue isolation method and maintained pure culture on PDA.

The isolates were challenge inoculated to highly susceptible rice variety, BPT-5204 (Samba Masuri). Based on lesion length and pathogenicity most virulent isolate was selected for our further work. Mandya HRL isolate of *R. solani* was observed to be more pathogenic than VC Farm Mandya because it caused high lesion length in a short period of incubation (Table 4 and Table 5).

Three different inoculation techniques were tested to identify best method of inoculation for quick and maximum disease induction. Among different methods of inoculation of pathogen agar block (plug) of pathogen inoculation was observed to be more efficient. In this method lesion length was highest up to 0.3 cm after 7 days of inoculation as compared to soil application of pathogen where lesion length was 0.3 cm after 30 days of inoculation.

Table 2: Morphological characterization of actinomycete isolates isolated from rice rhizosphere

Sl. No.	Isolate	Colony Characters		
		Morphology	**Pigmentation**	**Texture**
1	IABT-A1	White colour	Red colour	Metallic shiny colony
2	IABT-A2	White colour	White colour	Compact
3	IABT-A3	Black colour	Yellowish colour	Metallic shiny colony
4	IABT-A4	White colour	White colour	Solid substratum
5	IABT-A5	Black colour	White colour	Powdery
6	IABT-A6	Biscuit colour	White colour	Powdery
7	IABT-A7	Dark chocolate colour	Grey colour	Powdery
8	IABT-A8	Biscuit colour	Whitish Biscuit colour	Solid substratum
9	IABT-A9	Light greenish colour	White colour	Solid substratum
10	IABT-A10	Brownish colour	Yellow colour	Metallic shiny colony
11	IABT-A11	White colour	Red colour	Solid substratum

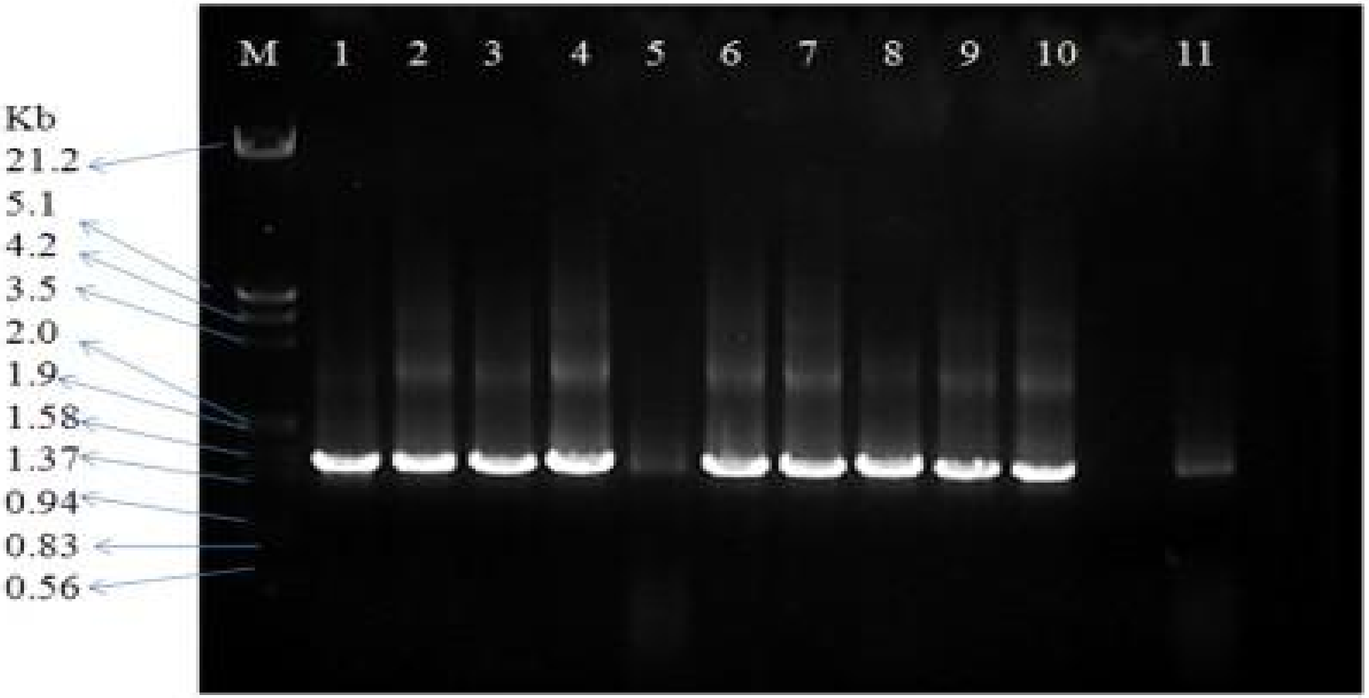


LABT-A2 IABT-A6

IABT-A7 IABT-A1

IABT-A11 IABT-A8

Plate 1. Colony morphology of actinomycete isolates



M-marker(HindIII double digest)
Actinomycetes isolates-1: IABT-A1, 2:IABT-A2, 3:IABT-A3, 4:IABT-A4, 5:IABT-A5, 6:IABT-A6, 7:IABT-A7, 8:IABT-A8, 9:IABT-A9, 10:IABT-A10, 11:IABT-A11

Plate 2. 16S rDNA amplification of eleven actionomycete isolates

Table 3a: ARDRA analysis of actinomycete isolates

Isolate Name	**IABT-A1**	**IABT-A2**	**IABT-A3**	**IABT-A4**	**IABT-A5**	**IABT-A6**	**IABT-A7**	**IABT-A8**	**IABT-A9**	**IABT-A10**	**IABT-A11**
Digestion with *Sau3AI*											
Results	=750bp	980-1350bp	=750bp	=750bp	=750bp	980-1350bp	980-1350bp	=750bp	=750bp	=750bp	=750bp
Branch	A	B	A	A	A	B	B	A	A	A	A
Digestion with *AsnI*											
Results	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb
Digestion with *KpnI*											
Results	410-470bp& 1-1.1kb	410-470& 1000-1100bp	410-470bp& 1-1.1kb	410-470bp& 1-1.1kb	410-470bp& 1-1.1kb			410-470bp& 1-1.1kb	410-470bp& 1-1.1kb	410-470bp& 1-1.1kb	410-470bp& 1-1.1kb
Digestion with *SphI*											
Results	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb	UC 1.5kb
Cook's no.	A2.1.2	*PstI*	A2.1.2	A2.1.2	A2.1.2	*PstI*	*PstI*	A2.1.2	A2.1.2	A2.1.2	A2.1.2
		UC 1.5kb				UC 1.5kb	UC 1.5kb				
		SnaBI				*SnaBI*	*SnaBI*				
		UC 1.5kb				UC 1.5kb	UC 1.5kb				
		B1.2.1.1.1				B1.2.1.1.1	B1.2.1.1.1				
Species	*Strepto.*	*Actinopolymorpha*	*Strepto.*	*Strepto.*	*Strepto.*	*Actinopolymorpha*	*Actinopolymorpha*	*Strepto.*	*Strepto.*	*Strepto.*	*Strepto.*

UC-Uncut, *Strepto.-Streptomyces* spp.

Table 3b: ARDRA analysis of actinomycete isolates

Sl. No.	Isolate name	Species
1	IABT-A2	*Actinopolymorpha spp*.
2	IABT-A6	
3	IABT-A7	
4	IABT-A1	*Streptomyces spp*
5	IABT-A3	
6	IABT-A4	
7	IABT-A5	
8	IABT-A8	
9	IABT-A9	
10	IABT-A10	
11	IABT-A11	

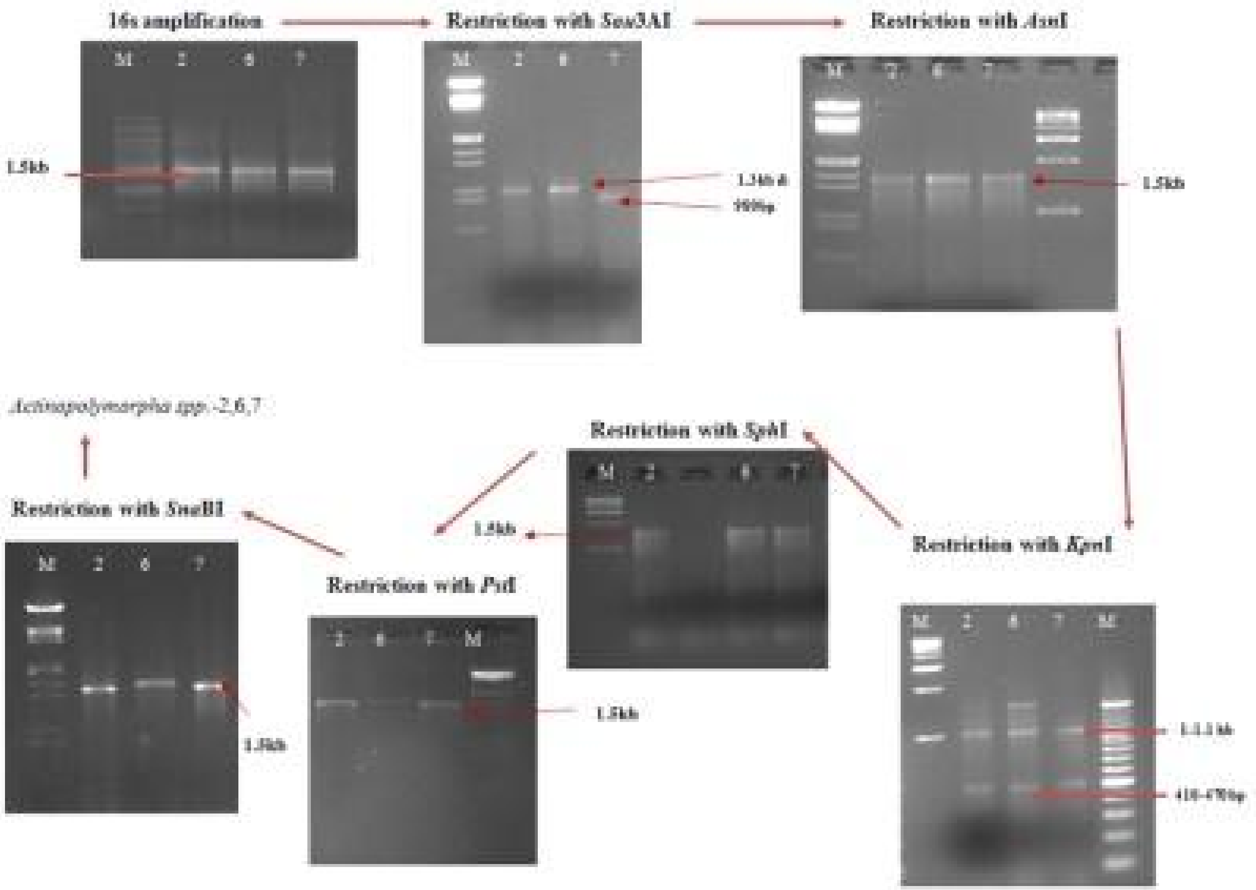


Plate 3. Identification of actinomycete isolates through rDNA restriction analysis (ARDRA)

Table 4: Methods of inoculation of *R. solani*

Method	No. of days after inoculation	Lesions length (cm)
Agar block	7	0.30
Liquid cultured mycelia ball	21	0.30
Soil treatment	30	0.30

Table 5: Relative lesion height on rice sheath by *R. solani* isolates

Pathogen isolates	No. of days after inoculation	Lesions length (cm)
Mandya HRL	15	0.3
	21	0.6
	30	1.2
VC Farm Mandya	15	0.2
	21	0.5
	30	1

Infection by target pathogen was confirmed by reisolating pathogen from infected diseased sheath and thus Koch's postulates were proved.

4.5 Screening of biocontrol agents against *R. solani* under *in vitro* condition

The biocontrol agents *Trichoderma spp.*, Fluorescent pseudomonad strains and strains of Pink pigmented facultative methylotrophs, *actinomycetes spp*.were evaluated against *Rhizoctonia solani* under *in vitro* condition and promising isolates were further evaluated under *in vivo* condition.

4.5.1 Evaluation of *Trichoderma spp.*against *R. solani*

The observations on the inhibition of mycelial growth of *Rhizoctonia solani* was recorded and tabulated in Table 6. The results revealed that all the thirteen isolates of *Trichoderma spp.* inhibited the growth of *R. solani* isolates compared to control. *T. harzianum* 1034 showed maximum inhibition of 69.33 per cent over control, significantly followed by *T. harzianum* 1040 and *T. harzianum* 1036 with per cent inhibition of 67.7 per cent and 66 per cent respectively. The inhibition of *R. solani* isolate Mandya HRL ranged from 62.23-73.11 per cent. *T. virens* 1002, *T. virens* 1010 and *T. harzianum* 1034 showed maximum inhibition of 73.11, 73.10 and 70.83 per cent respectively over the control (Fig.3) (Plate 4).

4.5.2 Evaluation of Fluorescent pseudomonad strains against *R. solani*

Dual culture studies of Fluorescent pseudomonad strains against *R. solani* revealed that the inhibition of mycelial growth of *R. solani* isolates Mandya HRL and VC Farm Mandya ranged from 83-100 per cent and 63-100 per cent respectively. Among the fifteen strains of Fluorescent pseudomonad, strain 19 was found to be highly effective in controlling both the isolates with 100 per cent inhibition. Many strains were found promising with inhibiton range of 90-100 per cent against both the isolates, as strain 6, 12, 22, 25, 30 and soy6 (Table 7, Fig. 4, Plate 5).

4.5.3 Evaluation of PPFM against *R. solani*

The Methylobacterial strain 38U significantly reduced the linear mycelial growth of *R. solani* isolates Mandya HRL to an extent of 83.33 per cent over control (Plate 6) followed by 42U and 26U showed 50 and 38.89 per cent inhibition in Mandya HRL isolate of pathogen respectively (Table 8). Remaining isolates of PPFM were not effective and showed no inhibition against the *R. solani* isolates. None of the tested PPFM strains were effective against *R. solani* isolate VC Farm Mandya.

4.5.4 Evaluation of actinomycete isolates against *R. solani*

Eleven isolates of actinomycete were screened against the test pathogen. All the tested isolates were found promising with inhibition range of 71.85-100 per cent against the pathogen. Actinomycete isolates IABT-A2, IABT-A7and IABT-A8 exhibited 100 per cent inhibition against all the three *R. solani* isolates tested in the study. The next best isolates were IABT-A3, IABT-A1 and IABT-A9 with mean per cent inhibition 99.62 per cent, 98.39 per cent and 98.27 per cent respectively. Based on per cent inhibition of *R. solani* isolates potent actinomycete isolates were selected for *in vivo* analysis (Table 9, Plate 7, Fig. 5).

4.6 Mass multiplication of potent bioagents for *in vivo* study

Total five isolates of *Trichoderma spp. viz.*, *T. virens* (1002), *T. virens* (1010), *T. harzianum* (1034), *T. harzianum* (1036) and *T. harzianum* (1040) were selected as potent isolates based on *in vitro* studies. Mass multiplication was done with autoclaved sorghum and sand mixture (2:1) and a good no.of spore per gram of culture of each isolates was recorded. In all further work the spore load of 7×10^6/ml was considered for study.

In case of Fluorescent pseudomonad five strains *viz.*, F. pseudomonad (12) (19) (20) (soy2) and (soy6) were selected as potent isolates. Mass multiplication was done. Cell count in King's B broth culture 55×10^8 cfu/ml was maintained at the time of application for *in vivo* study.

On the other hand in case of actinomycetes IABT-A1, IABT-A2, IABT-A6, IABT-A7 and IABT-A8 were taken as potent and mass multiplied in Starch Casein broth culture shown 10^8cfu/ml spore counting in each of culture which was used for rest of the studies.

4.7 Evaluation of bioagents against *R. solani* under glasshouse condition

All the potent isolates of biocontrol agents were tested under in vivo condition against *R. solani*.

Table 6: Evaluation of *Trichoderma* isolates against mycelial growth of *Rhizoctonia solani* isolates

Sl. No.	***Trichoderma* isolate**	**Per cent inhibition (mm)**		**Mean**
		Isolate- VC Farm Mandya	**Isolate-Mandya HRL**	
1	IABT 1002	64.77 * (53.62) ^{abcd}	73.11* (58.80) a	68.96
2	IABT 1006	52.6 (45.8) ^{e}	66.29 (54.53) ^{cde}	58.89
3	IABT 1010	60.44 (50.98) ^{cd}	73.10 (58.77) ^{a}	66.73
4	IABT 1016	63.7 (52.96) ^{abcd}	68.18 (55.67) ^{bcd}	65.94
5	IABT 1032	63.33 (52.51) ^{abcd}	66.66 (54.75) ^{cd}	64.81
6	IABT 1034	69.33 (56.33) ^{a}	70.83 (57.31) ^{ab}	70.04
7	IABT 1036	66 (54.29) ^{abc}	68.56 (55.90) ^{bcd}	67.24
8	IABT 1040	67.7 (55.42) ^{ab}	69.69 (56.63) ^{abc}	68.73
9	IABT 1041	64.88 (56.10) ^{a}	62.50 (52.25) ^{e}	65.69
10	IABT 1042	63.33 (52.73) ^{abcd}	65.15 (53.82) ^{de}	64.24
11	IABT 1043	58.5 (49.93) ^{d}	65.53 (54.05) ^{de}	62.02
12	IABT 1044	61.5 (51.64) ^{bcd}	67.04 (54.97) ^{bcd}	64.26
13	IABT 1046	63.77 (53.63) ^{abcd}	62.23 (52.08) ^{e}	63.52
14	Control	0.00	0.00	0.00
	S. Em ±	0.94	0.88	
	CD value (1%)	3.68	3.49	
	CV value	3.32	2.78	

* Figures in parentheses are arcsine transformed values

Mean with different letters are significantly different from each other (P<0.01) according to Duncan test

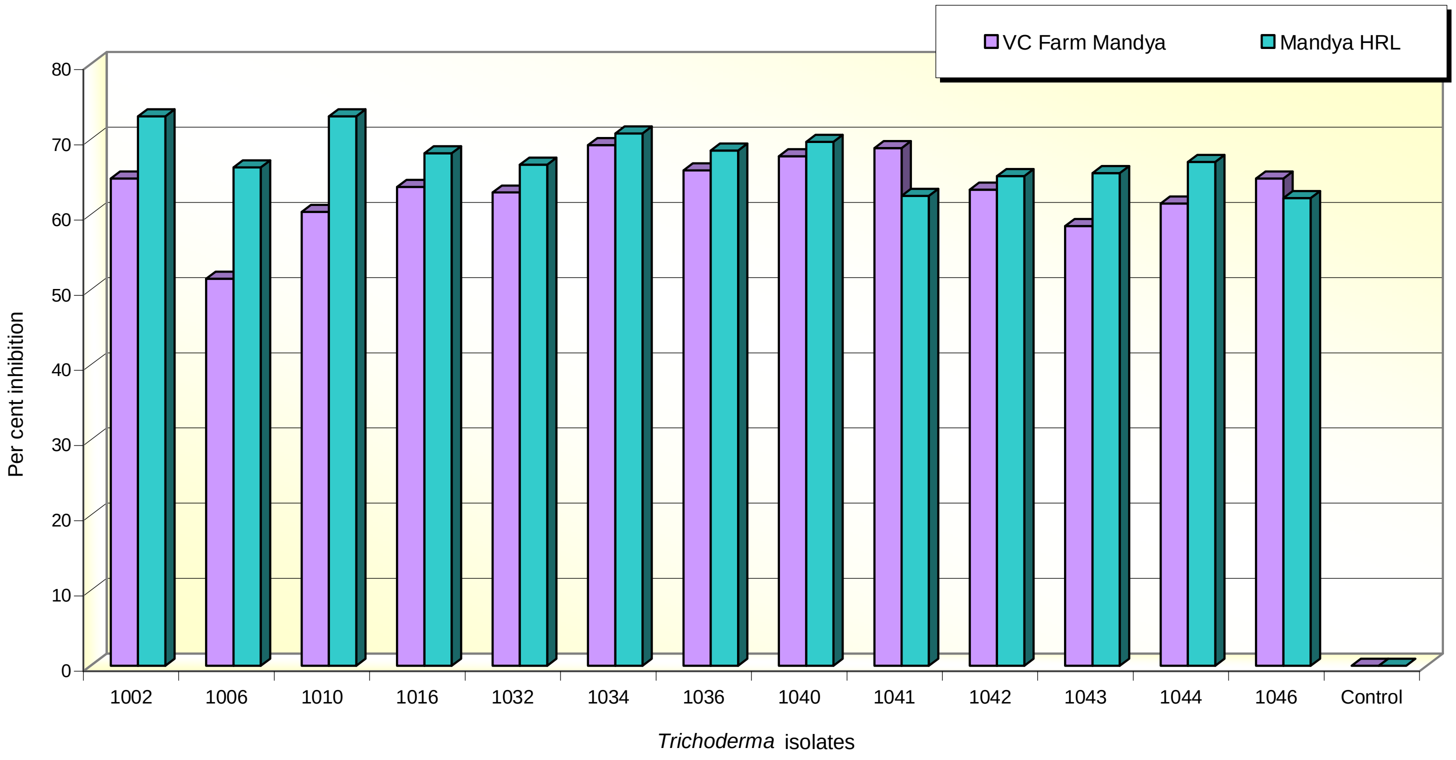
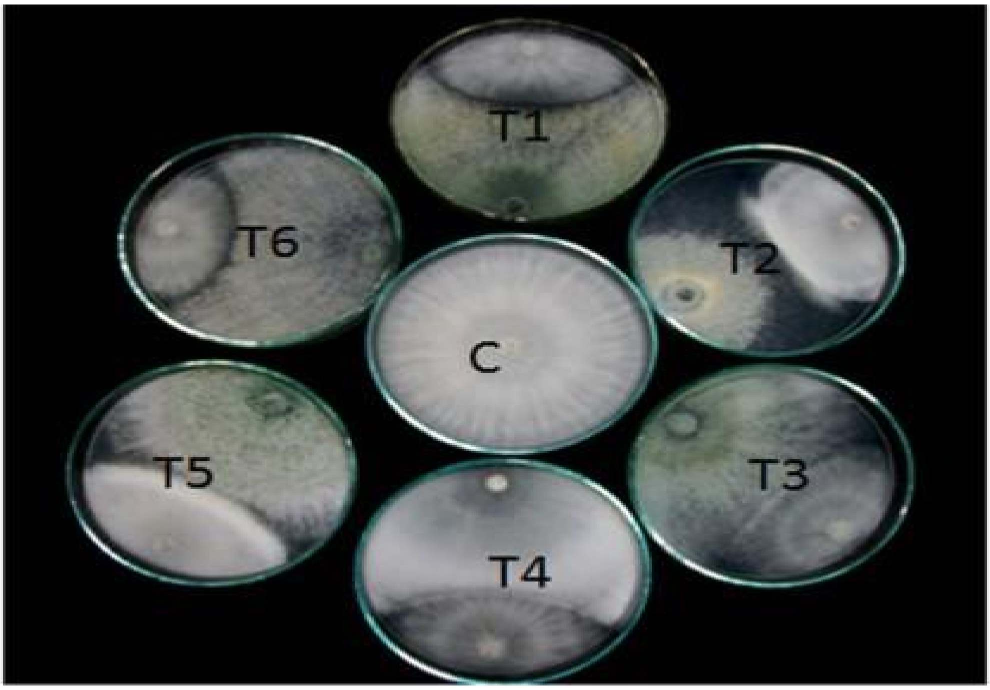


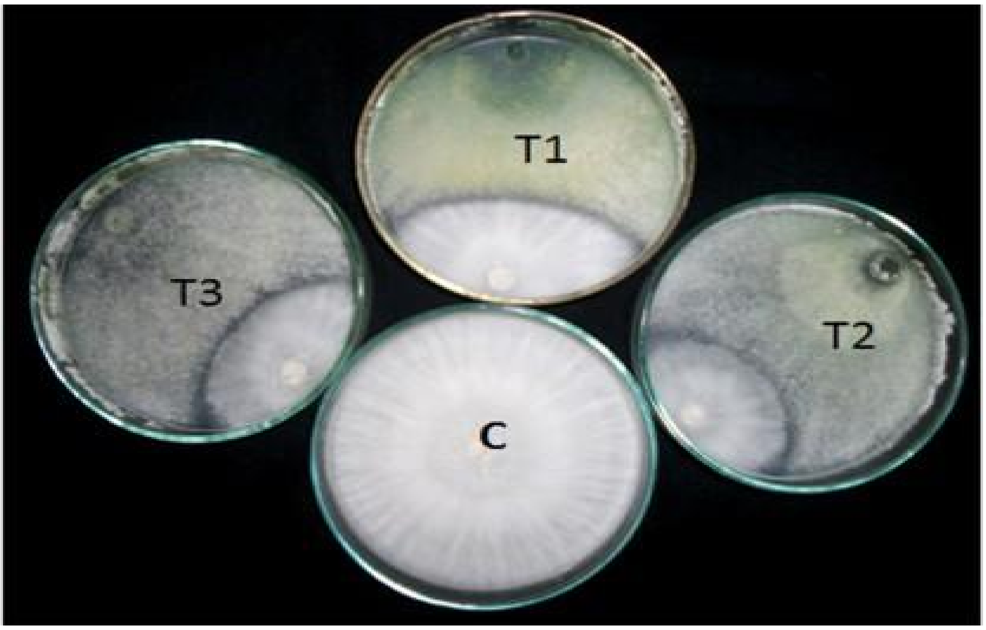
Fig. 3: *In vitro* evaluation of different Trichoderma isolates against *R. solani* isolates

Fig. 3:*In vitro* evaluation of different *Trichoderma* isolates against *R.solani* isolates



C-Control

Trichoderma isolates : T1-1036, T2-1041, T3-1010, T4-1046, T5-1042, T6-1034



C-Control

Trichoderma spp., T1-IABT 1034, T2-IABT 1040 T3-IABT 1036

Plate 4. Efficacy of Trichoderma isolates aginst R. solani

Table 7: Evaluation of Fluorescent pseudomonad strains against mycelial growth of *Rhizoctonia solani* isolates

Sl. No.	Fluorescent pseudomonad strains	Per cent inhibition (mm)		Mean
		Isolate-Mandya HRL	Isolate-VC Farm Mandya	
1	1	83.33 * (65.90) ^{f}	77.78 (61.87) ^{bcd}	80.55
2	6	84.81 (67.07) ^{ef}	97.04 (84.21) ^{a}	90.92
3	12	100 (90) ^{a}	96.30 (83.50) ^{a}	98.14
4	15	87.04 (68.90) ^{de}	70.74 (57.25) ^{cd}	78.89
5	19	100 (90) ^{a}	100 (90) ^{a}	100
6	20	88.89 (70.52) ^{cd}	97.04 (84.21) ^{a}	92.96
7	22	90.74 (72.29) ^{c}	81.11 (64.24) ^{bcd}	85.93
8	25	92.96 (74.62) ^{b}	78.89 (62.64) ^{bcd}	85.93
9	30	84.81 (67.06) ^{ef}	91.11 (72.65) ^{b}	87.96
10	Soy6	94.44 (76.36) ^{b}	81.11 (64.23) ^{bcd}	87.96
11	Soy2	88.89 (70.52) ^{cd}	87.04 (68.90) ^{bc}	87.78
12	Wht2	85.56 (67.69) ^{ef}	63.70 (52.99) ^{d}	74.63
13	Grnrt1	84.07 (66.52) ^{f}	65.56 (54.06) ^{d}	74.81
14	Safrrt1	88.52 (70.19) ^{d}	79.63 (63.33) ^{bcd}	84.07
15	IOF3	84.81 (67.07) ^{g}	68.15 (55.66) ^{d}	76.48
16	Control	0.00	0.00	0.00
	S. Em ± CD value (1%) CV value	0.45 1.78 1.17	2.75 10.68 7.49	

* Figures in parentheses are arcsine transformed values

Mean with different letters are significantly different from each other ($P<0.01$) according to Duncan test

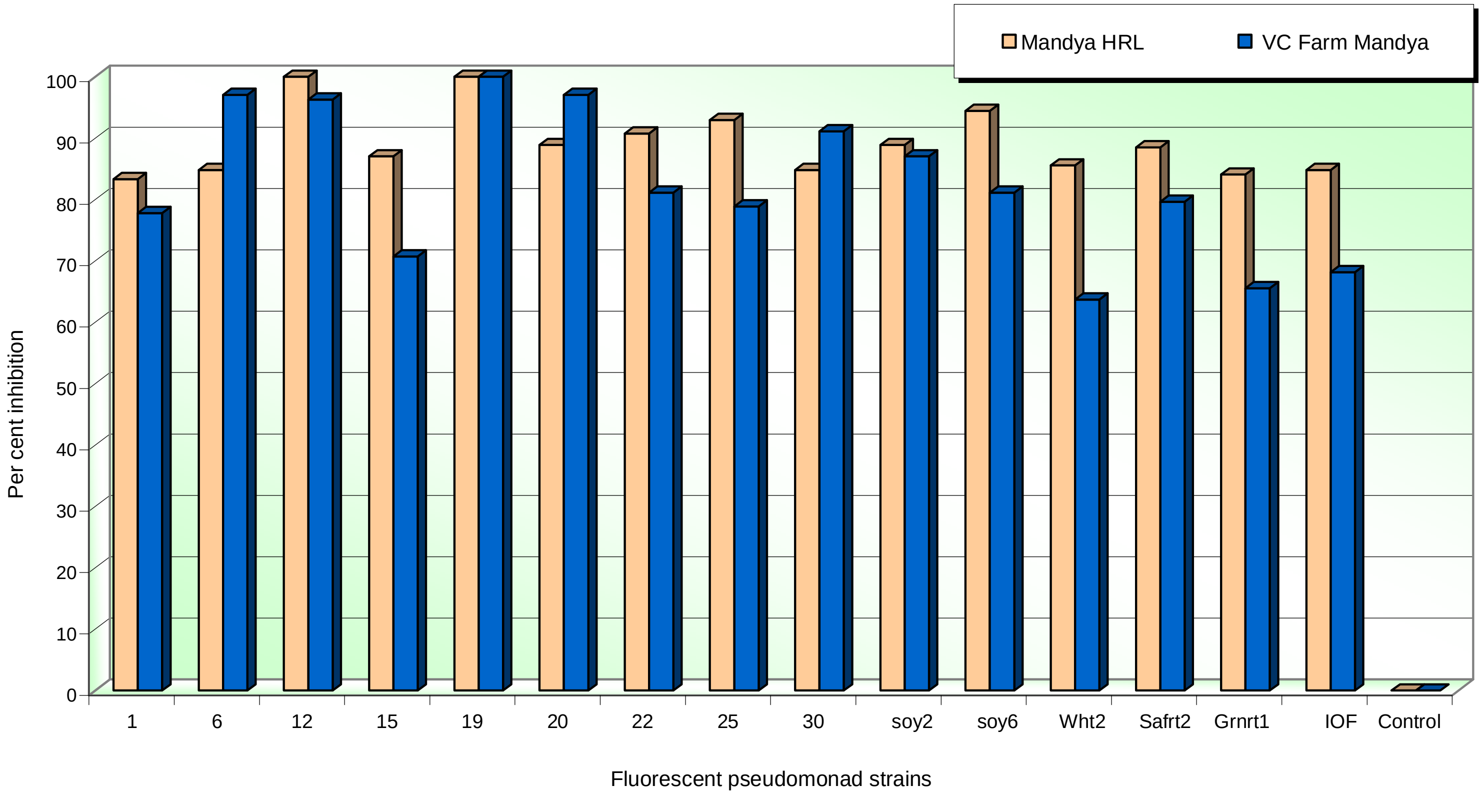
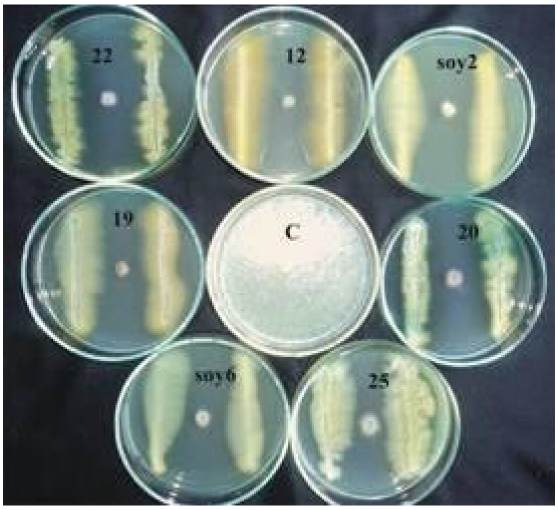
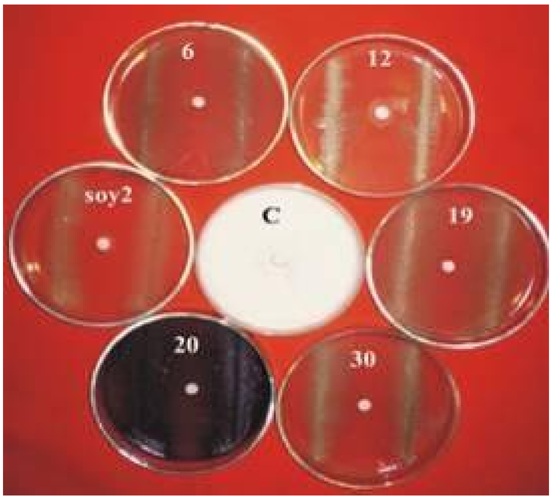


Fig. 4: ***In vitro*** **evaluation of Fluorescent pseudomonad strains against mycelial growth of** ***R. solani*** **isolates**

Fig. 4: In vitro evaluation of Fluorescent pseudomonad strains against mycelial growth of R. solani isolates



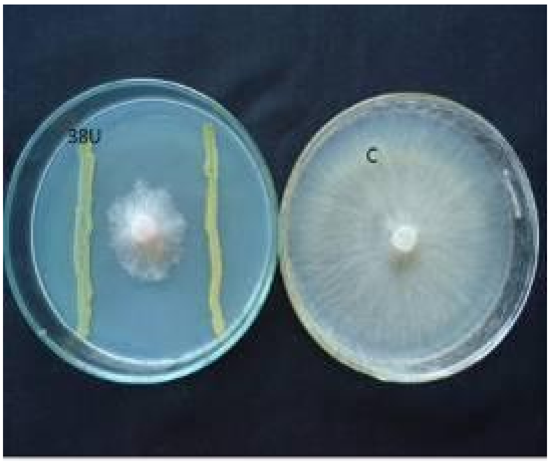
C-Control (Mandya HRL)



C-Control (VC Farm, Mandya)

Fluorescent pseudomonad strains-6, 12, 19,30,20, soy2, 22,25 and soy6

Plate. 5. Effecacy of Fluorescent pseudomonad against R. solani isolates



C-Control (Mandya HRL)

PPFM isolate-38U

Plate 6. Efficacy of PPFM against R.solani

Table 8: Evaluation of pink pigmented facultative methyllotrophs (PPFM) strains against mycelial growth *Rhizoctonia solani* isolates

Sl. No.	Pink pigmented facultative methyllotrophs (PPFM)	Per cent inhibition		
		Isolate-Mandya HRL	Isolate-VC Farm Mandya	Mean
1	27U	11.11* (19.47)	0 (0.00)	11.11
2	32	0 (0.00)	0 (0.00)	0.00
3	23A	3.33 (10.52)	0 (0.00)	3.33
4	10L	1.11 (6.05)	0 (0.00)	1.11
5	71Lin	5.56 (13.63)	0 (0.00)	5.56
6	H5	0 (0.00)	0 (0.00)	0.00
7	20A	0 (0.00)	0 (0.00)	0.00
8	75L	33.33 (35.26)	0 (0.00)	33.33
9	42U	50 (45.00)	0 (0.00)	50
10	26U	38.89 (38.58)	0 (0.00)	38.89
11	38U	83.33 (65.91)	0 (0.00)	83.33
12	Control	0.00	0.00	0.00
	Non significant			

* Figures in parentheses are arcsine transformed values

Table 9: Evaluation of isolates of actinomycete against mycelial growth of fungal pathogen *Rhizoctonia solani* isolates

Sl. No.	Actinomycete isolates	Per cent inhibition			Mean
		Isolate-Mandya HRL	Isolate-VC Farm Mandya	Isolate-Mugad	
1	IABT-A1	100 * (90) ^{a}	95.21 (77.34) ^{c}	100 (90) ^{a}	98.39
2	IABT-A2	100 (90) ^{a}	100 (90) ^{a}	100 (90) ^{a}	100
3	IABT-A3	100 (90) ^{a}	100 (90) ^{a}	98.88 (86.49) ^{a}	99.62
4	IABT-A4	76.30 (60.86) ^{c}	84.07 (66.48) ^{de}	94.81 (76.85) ^{b}	85.06
5	IABT-A5	84.07 (66.48) ^{b}	86.30 (68.27) ^{d}	91.11 (72.67) ^{bc}	87.16
6	IABT-A6	100 (90) ^{a}	95.93 (78.44) ^{bc}	100 (90) ^{a}	98.64
7	IABT-A7	100 (90) ^{a}	100 (90) ^{a}	100 (90) ^{a}	100
8	IABT-A8	100 (90) ^{a}	100 (90) ^{a}	100 (90) ^{a}	100
9	IABT-A9	100 (90) ^{a}	96.67 (79.57) ^{b}	98.14 (85.45) ^{a}	98.27
10	IABT-A10	74.44 (59.63) ^{d}	74.07 (59.39) ^{f}	89.25 (70.87) ^{bc}	79.25
11	IABT-A11	71.85 (57.95) ^{e}	82.22 (65.06) ^{e}	84.44 (66.77) ^{c}	79.50
12	Control	0.00	0.00	0.00	0.00
	S. Em ±	0.17	0.48	1.68	
	CD value (1%)	0.70	1.93	4.91	
	CV value	0.42	1.18	3.84	

* Figures in parentheses are arcsine transformed values

Mean with different letters are significantly different from each other ($P<0.01$) according to Duncan test

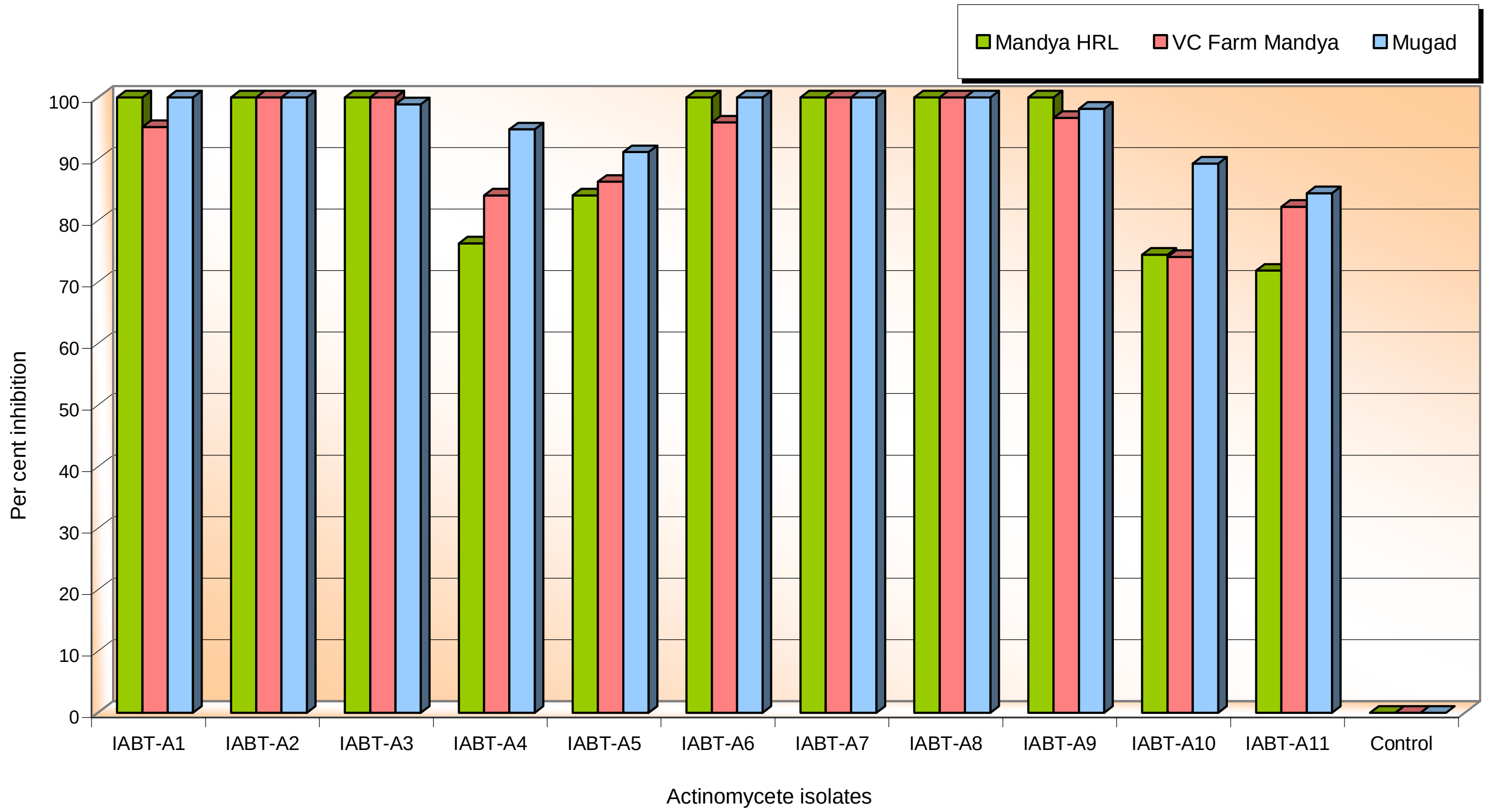
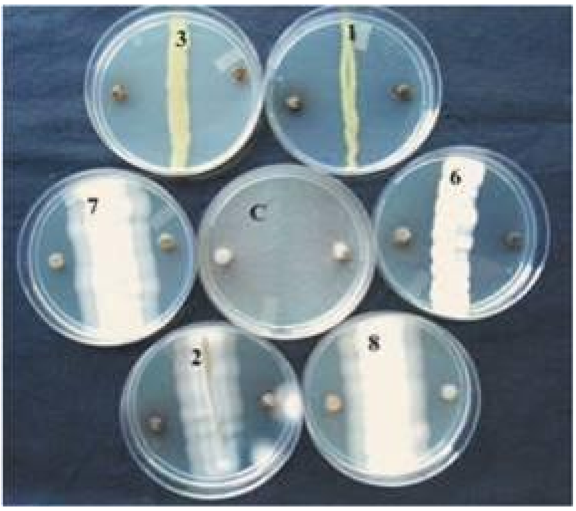


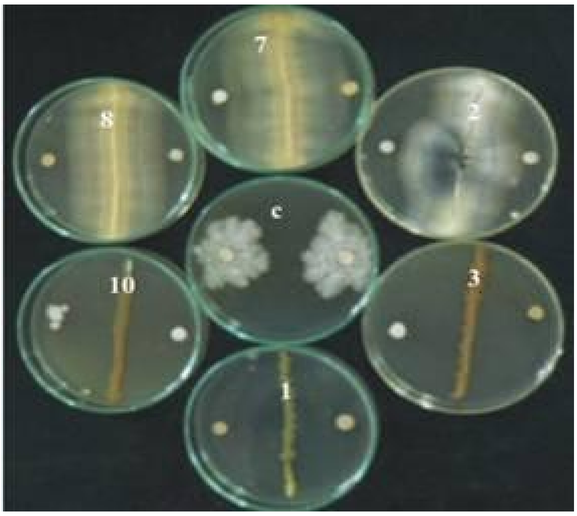
Fig. 5: ***In vitro*** **evaluation of actinomycete isolates against mycelial growth of** ***R. solani*** **isolate**

Fig. 5: In vitro evaluation of actinomycete isolates against mycelial growth of R. solani isolate



C-Control (Mandya HRL)

Actinomycete isolates-
3-IABT-A3,
1-IABT-A1,
6-IABT-A6,
8-IABT-A8,
2-IABT-A2,
7-IABT-A7,
10-IABT-A10,
4-IABT-A4



C-Control (VC Farm Mandya)

C-Control (Mugad)

Plate 7. Efficacy of Actinomycete spp. Against R.solani isolates

The disease parameters like lesion length and number of dried leaves due to disease were recorded. In addition growth parameters like shoot length, number of tillers, root length, dry weight of root of rice as influenced by different treatment of trichoderma, Fluorescent pseudomonad and actinomycetes with different strains were tabulated in Table 10 - 14.

4.7.1 Lesion length and no.of dried leaves due to sheath blight infection

The study revealed that in case of *Trichoderma* isolates, seed treatment of *T. harzianum* (1040) showed the lowest lesion length which was 0.84 cm at 40 DAS and 0.97 cm at 60 DAS with mean 0.90 cm followed by the *T. harzianum* (1036) in which mean lesion length of 0.94cm was recorded. The combined treatment (soil +seed +foliar application) of *T. harzianum* (1040) showed the lowest lesion length, which was 0.69 cm at 40 DAS and 0.70 cm at 60 DAS with mean 0.69cm. Lowest number of dried leaves due to pathogen infection was recorded in combined treatment with *T. harzianum* 1040 (2.33 leaves/plant) followed by seed treatment with *T. harzianum* 1040 (2.67 leaves/plant). The results on effect *Trichoderma spp*.on disease parameter are presented in Table 10, Fig.6a and 6b, Plate 8.

Five different F. pseudomonad strains were applied as seed treatment, soil treatment, foliar application and combination of these treatments. The effects of isolates and methods of delivery on disease parameter were studied under *in vivo* condition and presented in the Table 11. In case of Fluorescent pseudomonad strains, seed treatment of Fluorescent pseudomonad strain 19 showed the lowest lesion length which was 0.22 cm at 40 DAS and 0.62 cm at 60 DAS with mean 0.42 cm and number of dried leaves was 2.33 leaves/plant. The soil treatment with Fluorescent pseudomonad strain 19 showed the lowest lesion length which was 0.32 cm at 40 DAS and 0.70 cm at 60 DAS with mean 0.51 cm and number of dried leaves was 3 leaves/plant. Foliar spray of Fluorescent pseudomonad strain 19 showed the lowest lesion length which was 0.42 cm at 40 DAS and 0.86 cm at 60 DAS with mean 0.64 cm and number of dried leaves was 3.33 leaves/plant. Combined treatment with Fluorescent pseudomonad strain 19 showed the lowest lesion length of 0.11 cm at 40 DAS and 0.32 cm at 60 DAS with mean 0.22 cm and least number of dried leaves (1.67 leaves/plant) followed by combined treatment of Fluorescent pseudomonad strain 12 with lesion length of mean 0.41. The combined treatment with Fluorescent pseudomonad strain 19 was significantly superior with lowest lesion length and lowest number of dried leaves over treated control and other treatments (Fig.7, Plate 9 and 10).

Five different actinomycete isolates were evaluated under *in vivo* condition against *R. solani* infection and data on disease parameters, lesion length and no.of dried leaves are presented in Table 12 and depicted in Fig.8, Plate 11.

Among five actinomycete isolates, IABT-A7 was found best in all the delivery methods tested in this study with least lesion length and number of dried leaves. Seed treatment with IABT-A7 showed the lowest lesion length of 0.33 cm at 40 DAS and 0.38 cm at 60 DAS with mean value of 0.35 cm and number of dried leaves (2 leaves/plant). The combined treatment with IABT-A7 showed the lowest lesion length which was 0.11 cm at 40 DAS and 0.29 cm at 60 DAS with mean 0.19 cm and number of dried leaves was 1.33 leaves/plant. Isolate IABT-A7 was superior over other isolates in all the treatment methods tested with lowest lesion length and lowest number of dried leaves. In both soil treatment and foliar spray treatments, isolate IABT-A7 resulted least mean lesion length, 0.41 cm and 0.39 cm and number of dried leaves, 2.67 leaves/plant and 3 leaves/plant respectively.

4.7.2 Plant height

The data pertaining to effect on plant height are depicted in Table 13, 14 and Fig. 9a and 10a.

All biocontrol isolates enhanced the plant height compared to treated check. The highest height (79.85cm/plant) was observed in the plants inoculated with IABT-A7 isolate of actinomycete through the combined method of application at 40 DAS (Plate 12). This was followed by the seed treatment method with actinomycete isolate IABT-A7 (78.71cm/plant). In case of Fluorescent pseudomonad good plant height was observed in combined treatment of F. pseudomonad 19 (61.12cm/plant) over the healthy plant and treated control.

At 60 DAS, combination methods resulted in the highest plant height in actinomycete isolate IABT-A7 (80.89 cm/plant). The second best treatment was the seed treatment followed by soil and foliar spray treatment of actinomycete isolates IABT-A7 among five different isolates.

Table 10: Effect of different delivery methods of *Trichoderma* isolates on lesion length of disease, no.of tillers per plant, no.of dried leaves per plant of rice at different intervals of time

Tr. No.	Treatment	Disease parameters				Growth parameters
		Lesion length (cm)			No. of dried leaves per plant (40 DAS)	No.of tillers per plant (40 DAS)
		40 DAS	60 DAS	Mean		
T1	Seed treatment (1002)	1.87	1.89	1.88	5.33	1.00
T2	Seed treatment (1010)	1.38	1.40	1.39	4.67	1.33
T3	Seed treatment (1034)	1.14	1.17	1.15	3.67	2.33
T4	Seed treatment (1036)	0.93	0.95	0.94	3.67	2.67
T5	Seed treatment (1040)	0.84	0.97	0.90	2.67	3.33
Mean		1.23	1.28	1.25	4	2.13
T6	Combined treatment (1002)	1.75	1.83	1.79	5.00	2.00
T7	Combined treatment (1010)	1.17	1.25	1.21	4.33	1.67
T8	Combined treatment (1034)	0.97	1.14	1.05	3.33	2.67
T9	Combined treatment (1036)	0.95	0.99	0.97	3.33	3.00
T10	Combined treatment (1040)	0.69	0.70	0.69	2.33	3.67
Mean		1.11	1.18	1.14	3.67	2.6
T11	Pathogen (treated control)	3.83	4.27	4.05	7.67	1.33
	S. Em ±	0.18	0.06		0.36	0.28
	CD value (5%)	0.52	0.18		1.06	0.83
	CV value	21.91	7.19		15.00	21.66

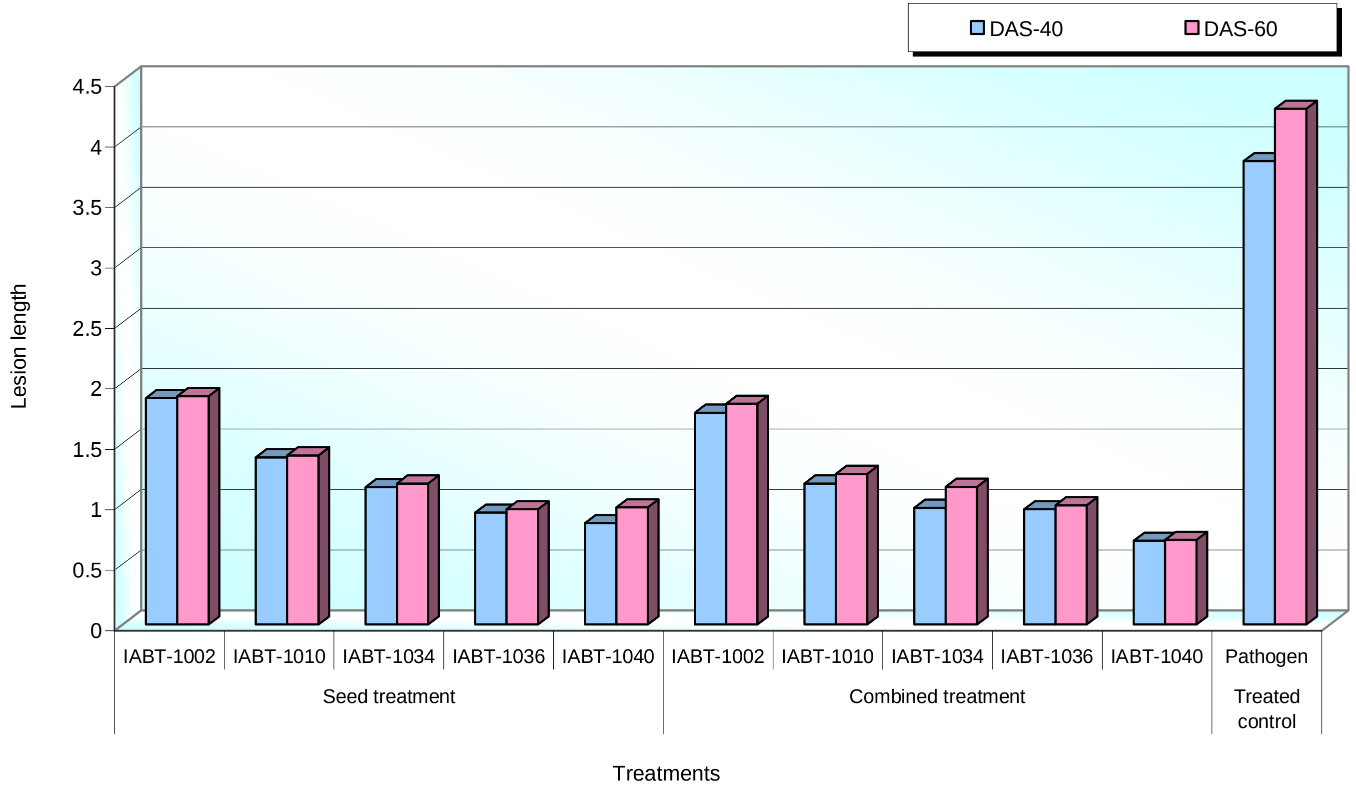


Fig.6a: Effect of different delivery methods of *Trichoderma* isolates on lesion length of disease

Fig. 6a: Effect of different delivery method of Trichoderma isolates on lesion length of disease

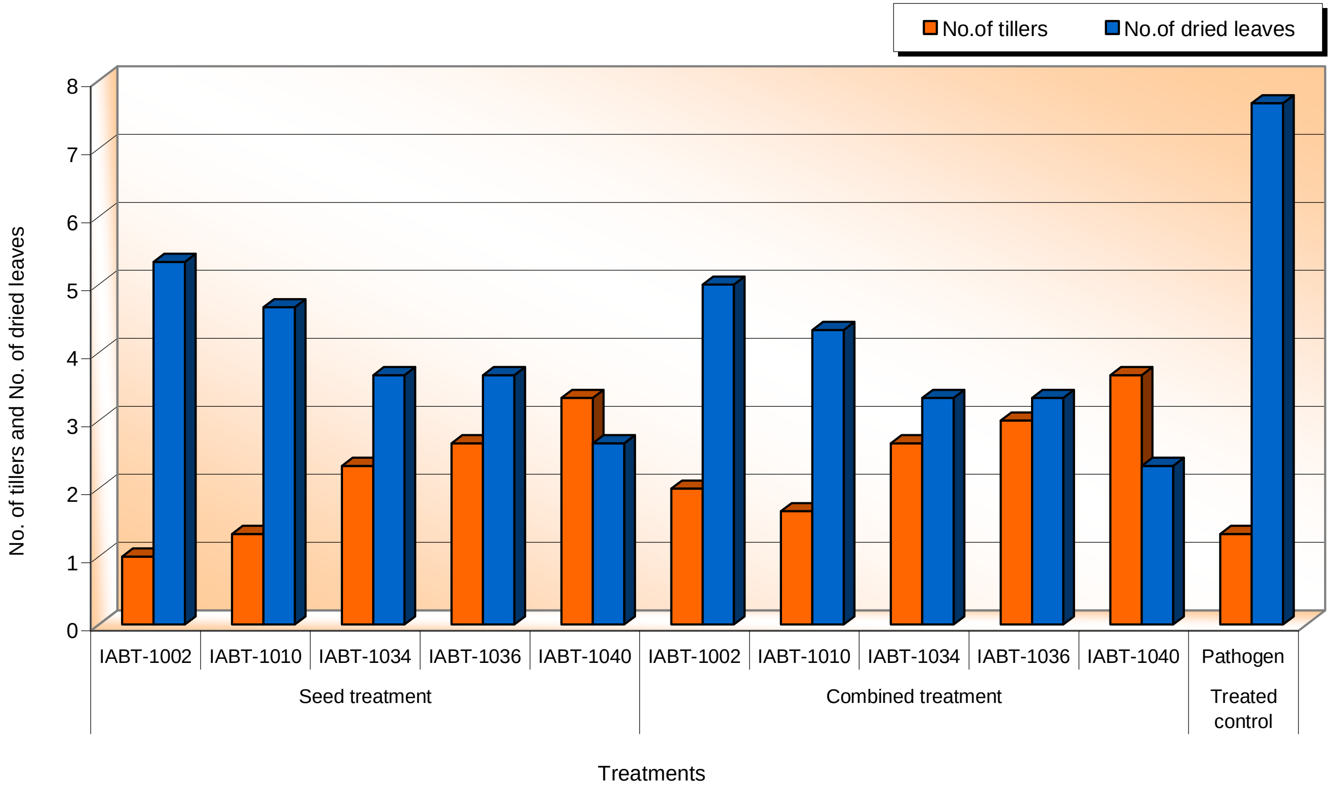


Fig. 6b: Effect of different delivery methods of *Trichoderma* isolates on Number of tillers and Number of dried leaves

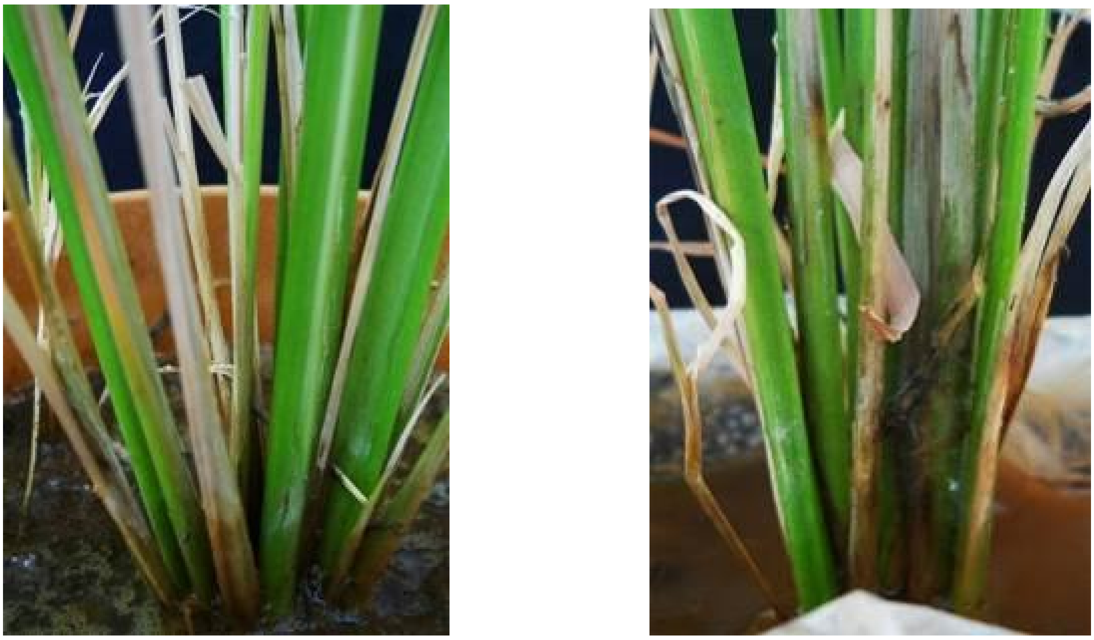
Fig. 6b: Effect of different delivery method of Trichoderma isolates on Number of tillers and Number of dried leaves

Lesion length due to sheath blight infection

Combine treatment of
Trichoderma isolate: 1040

Pathogen treatment

Plate 8. Lesion length in Trichoderma isolate: 1040 treatment



Combine treatment of
pseudomonad-19

Pathogen treatment

Plate 9. Lesion length in pseudomonad-19 treatment

Table 11: Effect of different delivery methods of strains of Fluorescent pseudomonad on disease parameters at different intervals of time

Tr. No.	Treatment	Lesion length (cm)			No. of dried leaves per plant (40 DAS)
		40 DAS	60 DAS	Mean	
T1	Seed treatment (12)	0.30	0.74	0.52	3.33
T2	Seed treatment (19)	0.22	0.62	0.42	2.33
T3	Seed treatment (20)	0.33	0.83	0.58	3.33
T4	Seed treatment (soy2)	0.53	0.87	0.70	4.33
T5	Seed treatment (soy6)	0.79	0.94	0.86	3.67
Mean		0.43	0.8	0.62	3.39
T6	Soil treatment (12)	0.37	0.87	0.62	3.33
T7	Soil treatment (19)	0.32	0.70	0.51	3.00
T8	Soil treatment (20)	0.39	1.03	0.71	3.67
T9	Soil treatment (soy2)	0.63	1.26	0.94	3.67
T10	Soil treatment (soy6)	0.89	1.01	0.95	3.33
Mean		0.52	0.97	0.75	3.4
T11	Foliar spray treatment (12)	0.47	1.17	0.82	4.33
T12	Foliar spray treatment (19)	0.42	0.86	0.64	3.33
T13	Foliar spray treatment (20)	0.57	1.27	0.92	3.67
T14	Foliar spray treatment (soy2)	0.87	1.57	1.22	4.00
T15	Foliar spray treatment (soy6)	1.38	1.90	1.64	3.67
Mean		0.74	1.35	1.048	3.8
T16	Combined treatment (12)	0.15	0.67	0.41	2.33
T17	Combined treatment (19)	0.11	0.32	0.22	1.67
T18	Combined treatment (20)	0.22	0.70	0.46	3.00
T19	Combined treatment (soy2)	0.44	0.92	0.68	3.33
T20	Combined treatment (soy6)	0.64	0.90	0.77	2.33
Mean		0.54	0.91	0.72	2.53
T21	Pathogen (treated Control)	3.8	4.83	4.31	7.67
T22	Healthy (Untreated control)	0.00	0.00	0.00	0.00
	S. Em ± CD value (5%) CV value	0.04 0.12 11.30	0.13 0.39 20.84		0.39 1.13 19.76

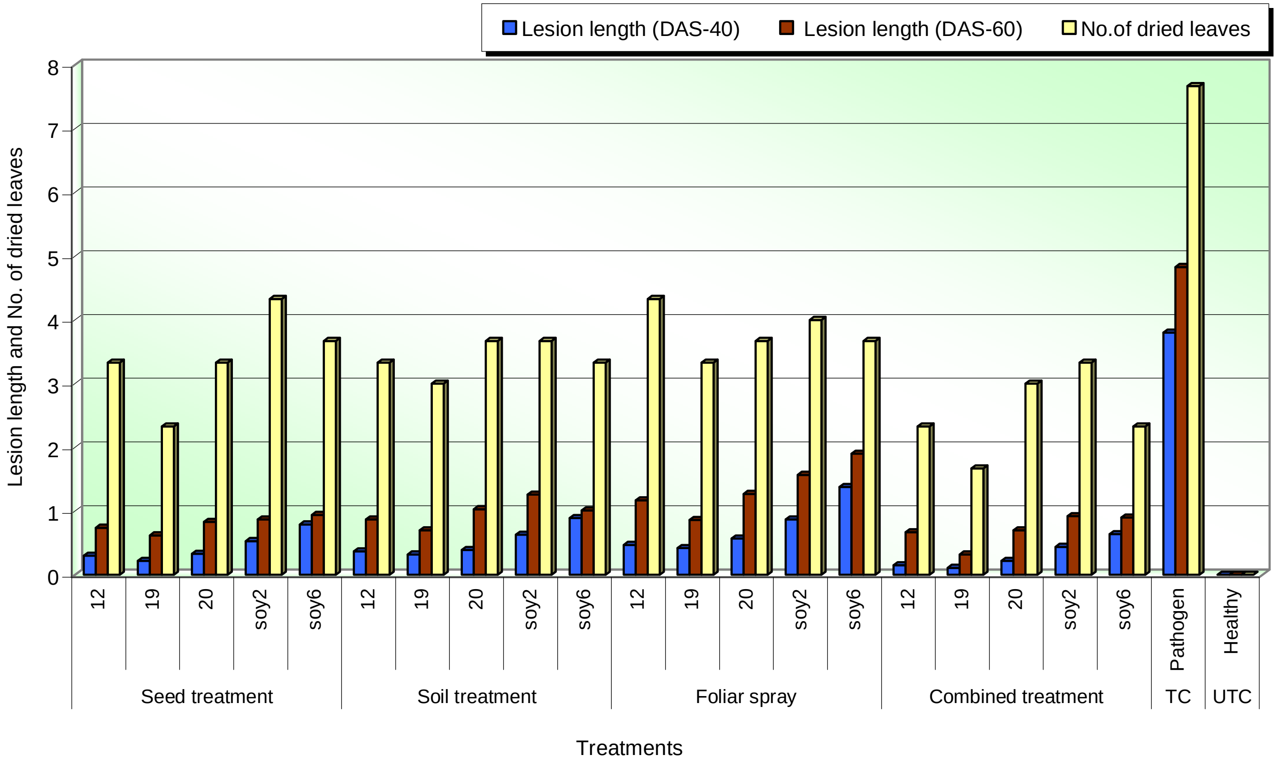


Fig.7: Effect of different delivery methods of Fluorescent pseudomonad strains on disease parameters at different time intervals

Legend

TC-Treated control, UTC-Untreated control

Fig.7: Effect of different delivery methods of Fluorescent pseudomonad strains on disease parameters at different time intervals

Plate 10. Effect of pseudomonad strain 19 on root growth

Table 12: Effect of different delivery methods of isolates of actinomycete on disease parameters at different intervals of time

Tr. No.	Treatment	Lesion length (cm)			No. of dried leaves per plant
		40 DAS	60 DAS	Mean	(40 DAS)
T1	Seed treatment (IABT-A1)	0.60	0.82	0.71	4.00
T2	Seed treatment (IABT-A2)	0.61	0.74	0.68	3.00
T3	Seed treatment (IABT-A6)	0.37	0.55	0.46	3.00
T4	Seed treatment (IABT-A7)	0.33	0.38	0.35	2.00
T5	Seed treatment (IABT-A8)	0.79	0.77	0.78	3.33
Mean		0.54	0.65	0.59	3.06
T6	Soil treatment (IABT-A1)	0.49	0.81	0.65	3.33
T7	Soil treatment (IABT-A2)	0.39	0.70	0.55	3.33
T8	Soil treatment (IABT-A6)	0.36	0.62	0.49	3.00
T9	Soil treatment (IABT-A7)	0.31	0.51	0.41	2.67
T10	Soil treatment (IABT-A8)	0.56	0.81	0.68	3.00
Mean		0.42	0.69	0.56	3.07
T11	Foliar spray treatment (IABT-A1)	0.42	1.17	0.79	3.67
T12	Foliar spray treatment (IABT-A2)	0.37	0.78	0.57	3.33
T13	Foliar spray treatment (IABT-A6)	0.27	0.67	0.47	4.00
T14	Foliar spray treatment (IABT-A7)	0.24	0.55	0.39	3.00
T15	Foliar spray treatment (IABT-A8)	0.71	0.94	0.82	3.33
Mean		0.40	0.82	0.61	3.46
T16	Combined treatment (IABT-A1)	0.33	0.73	0.53	3.00
T17	Combined treatment (IABT-A2)	0.22	0.63	0.42	2.67
T18	Combined treatment (IABT-A6)	0.17	0.41	0.29	2.00
T19	Combined treatment (IABT-A7)	0.11	0.28	0.19	1.33
T20	Combined treatment (IABT-A8)	0.44	0.68	0.56	2.00
Mean		0.27	0.48	0.37	2.2
T21	Pathogen (treated Control)	3.63	4.33	3.98	7.67
T22	Healthy (Untreated control)	0.00	0.00	0.00	0.00
	S. Em ±	0.10	0.08		0.38
	CD value (5%)	0.28	0.23		1.09
	CV value	31.28	16.55		21

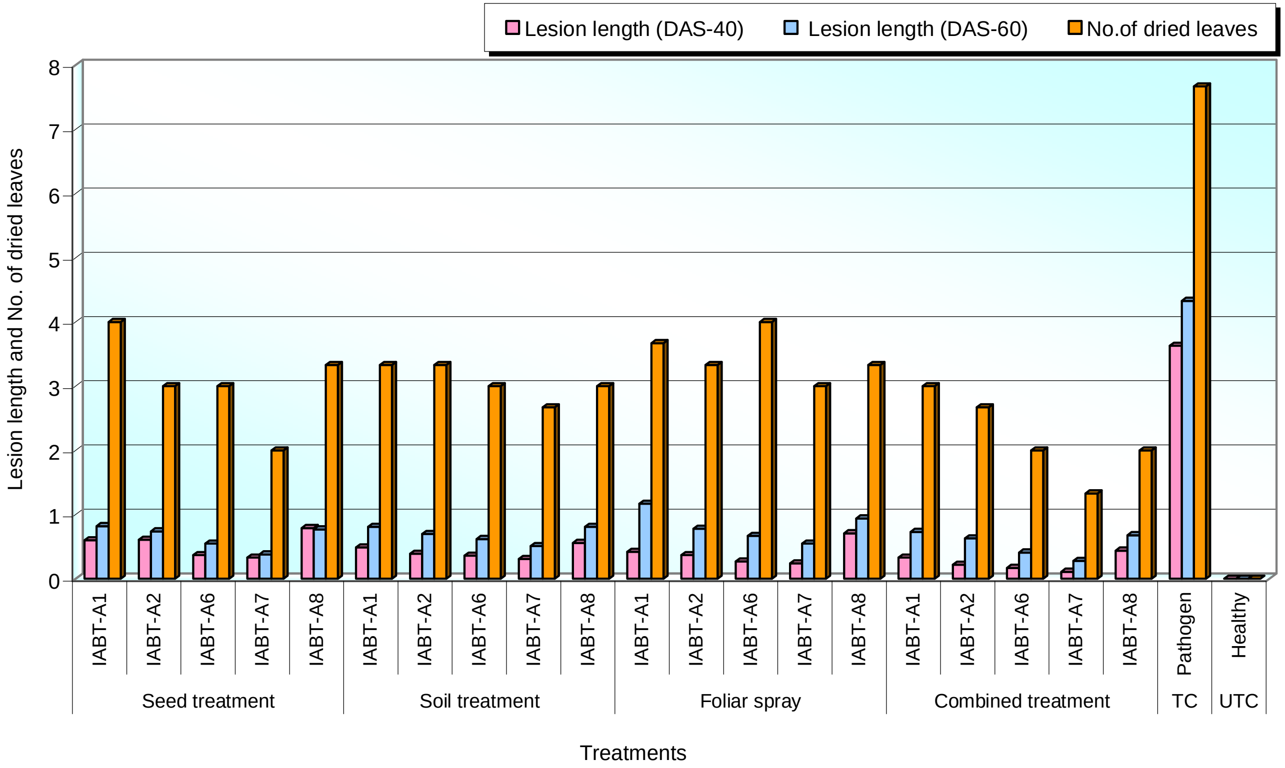


Fig.8: Effect of different delivery methods of actinomycete isolates on disease parameters at different intervals of time

Legend

TC-Treated control, UTC-Untreated control

Fig.8: Effect of different delivery methods of actinomycete isolates on disease parameters at different intervals of time

Combined treatment **Only pathogen treatment**

Plate 11. Lesion length in actinomycete isolate IABT-A7 treatment

1-Combined treatment **4-Foliar spray**

2-Seed treatment **5-Control (pathogen)**

3-Soil treatment **6-Control (healthy)**

Plate 12. Effect of actinomycete isolate IABT-A7 on plant height

Table 13: Effect of Fluorescent pseudomonad strains on plant growth parameters in presence of *R. solani*

Tr. No.	Treatment	Plant height (cm)			Root length (cm) (75 DAS)	Dry wt. of root per plant (gm)	No. of tillers per plant (40 DAS)
		40 DAS	60 DAS	Mean			
T1	Seed treatment (12)	52.53	53.03	52.78	41.71	0.50	2.67
T2	Seed treatment (19)	52.26	53.29	52.77	38.47	1.06	3.33
T3	Seed treatment (20)	44.83	45.84	45.33	39.59	1.02	2.33
T4	Seed treatment (soy2)	43.68	44.01	43.84	33.55	0.76	2.00
T5	Seed treatment (soy6)	47.74	48.41	48.07	30.60	0.61	1.67
Mean		48.20	48.91	48.56	36.78	0.79	2.4
T6	Soil treatment (12)	47.47	48.17	47.82	40.07	1.08	2.67
T7	Soil treatment (19)	48.64	49.11	48.87	35.97	1.26	3.67
T8	Soil treatment (20)	43.19	43.52	43.35	38.90	0.90	2.33
T9	Soil treatment (soy2)	41.64	42.31	41.97	26.53	0.53	1.33
T10	Soil treatment (soy6)	42.99	43.36	43.17	25.07	0.47	1.67
Mean		44.78	45.29	45.04	33.31	0.85	2.33
T11	Foliar spray treatment (12)	36.19	36.85	36.52	28.03	0.40	2.33
T12	Foliar spray treatment (19)	44.71	45.05	44.88	27.60	0.70	3.00
T13	Foliar spray treatment (20)	37.99	38.36	38.17	24.40	0.32	2.00
T14	Foliar spray treatment (soy2)	44.47	44.84	44.65	24.47	0.27	1.33
T15	Foliar spray treatment (soy6)	47.77	48.14	47.95	20.27	0.20	1.67
Mean		42.23	42.64	42.44	24.95	0.38	2.07
T16	Combined treatment (12)	52.40	53.47	52.93	37.32	2.07	3.33
T17	Combined treatment (19)	61.12	62.18	61.65	39.50	2.57	4.00
T18	Combined treatment (20)	56.25	57.41	56.83	34.20	1.66	2.67
T19	Combined treatment (soy2)	46.61	47.37	46.99	32.70	0.75	1.67
T20	Combined treatment (soy6)	52.15	52.61	52.38	28.57	0.58	2.33
Mean		53.71	54.61	54.16	34.45	1.53	2.8
T21	Pathogen (treated Control)	36.33	37.07	36.7	23.77	0.44	1.33
T22	Healthy (Untreated control)	37.63	38.26	37.94	31.83	0.60	2.33
	S. Em ± CD value (5%) CV value	0.53 1.53 2.01	0.50 1.42 1.85		0.73 2.10 4.00	0.05 0.15 10.89	0.30 0.85 22.23

Table 14: Effect of different delivery methods of isolates of actinomycete on growth parameters at different intervals of time

Tr. No.	Treatment	Plant height (cm)			Root length (cm) 75 DAS	Dry wt. of root (gm)	No. of tillers per plant (40 DAS)
		40 DAS	60 DAS	Mean			
T1	Seed treatment (IABT-A1)	45.73	46.77	46.25	24.10	1.21	2.33
T2	Seed treatment (IABT-A2)	55.91	56.25	56.08	38.50	2.98	3.33
T3	Seed treatment (IABT-A6)	67.56	68.89	68.22	36.17	2.38	4.00
T4	Seed treatment (IABT-A7)	78.71	79.93	79.32	37.93	3.18	4.67
T5	Seed treatment (IABT-A8)	48.86	49.82	49.34	35.50	1.88	2.67
Mean		59.35	60.33	59.84	34.44	2.32	3.4
T6	Soil treatment (IABT-A1)	35.15	36.17	35.66	23.37	1.52	2.00
T7	Soil treatment (IABT-A2)	51.11	52.15	51.63	30.60	2.22	2.67
T8	Soil treatment (IABT-A6)	64.20	65.32	64.76	37.23	1.82	3.33
T9	Soil treatment (IABT-A7)	74.40	75.31	74.85	38.50	1.81	4.00
T10	Soil treatment (IABT-A8)	50.77	51.17	50.97	29.50	2.08	2.33
Mean		55.12	56.02	55.57	31.84	1.89	2.86
T11	Foliar spray treatment (IABT-A1)	41.37	42.04	41.70	20.33	2.13	1.67
T12	Foliar spray treatment (IABT-A2)	45.55	46.61	46.08	28.10	2.55	2.33
T13	Foliar spray treatment (IABT-A6)	61.35	62.51	61.93	31.63	1.52	2.67
T14	Foliar spray treatment (IABT-A7)	73.83	74.93	74.38	32.60	1.68	3.00
T15	Foliar spray treatment (IABT-A8)	49.46	50.69	50.07	27.00	1.53	2.00
Mean		54.31	55.35	54.83	27.93	1.88	2.33
T16	Combined treatment (IABT-A1)	49.80	50.73	50.26	30.43	1.90	2.67
T17	Combined treatment (IABT-A2)	60.50	61.30	60.9	38.23	2.89	4.00
T18	Combined treatment (IABT-A6)	70.91	71.25	71.08	37.57	3.59	4.67
T19	Combined treatment (IABT-A7)	79.85	80.89	80.37	40.30	3.61	6.33
T20	Combined treatment (IABT-A8)	51.47	52.40	51.93	34.40	2.45	3.67
Mean		62.51	63.31	62.91	36.18	2.88	4.27
T21	Pathogen (treated Control)	37.91	38.59	38.25	28.13	1.88	1.67
T22	Healthy (Untreated control)	39.30	39.70	39.5	29.43	1.96	2.33
	CD value (5%) S. Em ± CV value	1.21 0.42 1.31	1.18 0.41 1.26		0.99 0.34 1.87	0.09 0.03 2.65	0.95 0.33 18.58

In case of F. pseudomonad combined treatment of F. pseudomonad 19 were shown good plant height as (62.18cm/plant) followed by seed treatment with 19 (52.77cm), soil treatment (48.87cm), foliar treatment (44.88cm) and combined treatment (61.65cm). Compared to other isolates combined treatment of IABT-A7 isolate was significantly superior over other treatments.

4.7.3 Number of tillers

Application of biocontrol agents increased the number of tillers/ plant compared to check (Table 13, 14). Among *Trichoderma* isolates, application of *T. harzianum* 1040 as combined treatment increased the number of tillers to the maximum extent as compared to other isolates. This was followed by seed treatment with *T. harzianum* 1040 (3.33 per plant). In case of Fluorescent pseudomonad strains, number of tillers was maximum in the combined treatment method of Fluorescent pseudomonad 19 (4 per plant), followed by soil treatment method of Fluorescent pseudomonad 19 (3.67 per plant) and the least number of tillers was recorded in the untreated control followed by pathogen inoculated treatment as (2.33 and 1.33 per plant) respectively (Fig. 9b).

Among actinomycete isolates, IABT-A7 incresed the number of tillers to the maximum when applied as combined treatment (6.33 per plant). Next best was combined treatment of IABT-A6 and seed treatment of IABT-A7 with tillers 4.67 per plant. The least number of tillers was seen in pathogen inoculated treatment 1.67 per plant (Fig. 10b).

4.7.4 Root length

Significant differences of root length of rice plant was observed due to various delivery methods and different bioagents.

Root length was maximum in seed treatment of F. pseudomonad 12 as (41.71cm) followed by combined treatment of F. pseudomonad 19 as (39.50 cm). And lower root length was recorded in foliar spray treatment of F. pseudomonad soy 6 as (20.27cm) (Table 13, Fig. 9a, Plate 10).

In case of actinomycetes, in seed treatment method maximum root length was recorded with isolate IABT-A2 (38.50 cm) which was significantly on par with isolate IABT-A7 with (37.93 cm). In soil treatment method, IABT-A7 increased the root length upto 38.50 cm significantly. However when IABT-A7 was applied as combined treatment it could increased the root length to the maximum extent (40.30 cm). and foliar spray treatment of actinomycete IABT-A7 as (20.33cm) which was on par with the healthy plant and only pathogen treated control (Table 14, Fig. 10a,Plate 14).

4.7.5 Dry weight of root

In case of pseudomonad strains, combined treatment of F. pseudomonad 19 was significantly superior over other strains with maximum dry weight of root as 2.57g/plant. The least root dry weight was recorded in pathogen treated control as 0.44g/plant (Table 13, Fig.9b).

The root dry weight of rice crop was found to increase significantly due to combined treatment of actinomycete isolate IABT-A7 (3.61g/plant) and combined treatment of actinomycete isolate IABT-A6 (3.59g/plant) compared to treated control(1.88 g/plant). The next best treatments were seed treatment of actinomycete IABT-A7 (3.18g/plant) and seed treatment of actinomycete (IABT-A2 2.98g/ plant) (Table14, Fig.10b).

The results on effect of different bioagents when applied as combined treatment (seed treatment+ soil application + foliar spray) against *R.solani* is compiled and presented in the Table 15. Among the three bioagents, *Actinopolymorpha spp.*(actinomycete IABT-A7) significantly reduced sheath blight infection with 0.19 cm lesion length and 1.33 dry leaves/ plant. Additionally, application of this isolate promoted plant growth with highest plant height (80.37 cm) and dry root weight 3.59 g/plant compared to pseudomonad strain 19. Hence, *Actinopolymorpha spp.*(actinomycete IABT-A7) was selected to understand the defence pathways operating in rice-*R. solani* - bioagent interaction.

4.8 Total RNA isolation and RNA integrity

For expression profiling of defense gene involved in present study, bioagent actinomycete isolate IABT-A7 was selected as potent based on the results obtained under *in vitro* and *in vivo* evaluation. The total RNA was isolated from different with isolate actinomycete viz, combined treatment, seed treatment, only bioagent treatment, treated check and healthy plant using TRIZOL reagent (Invitrogen, San Diego USA) as explained in materials and methods.

Fig.9a: Effect of different delivery methods of Fluorescent pseudomonad strains on plant height and root length at different intervals of time

Legend

TC-Treated control, UTC-Untreated control

Fig.9a: Effect of different delivery methods of Fluorescent pseudomonad strains on plant height and root length at different intervals of time

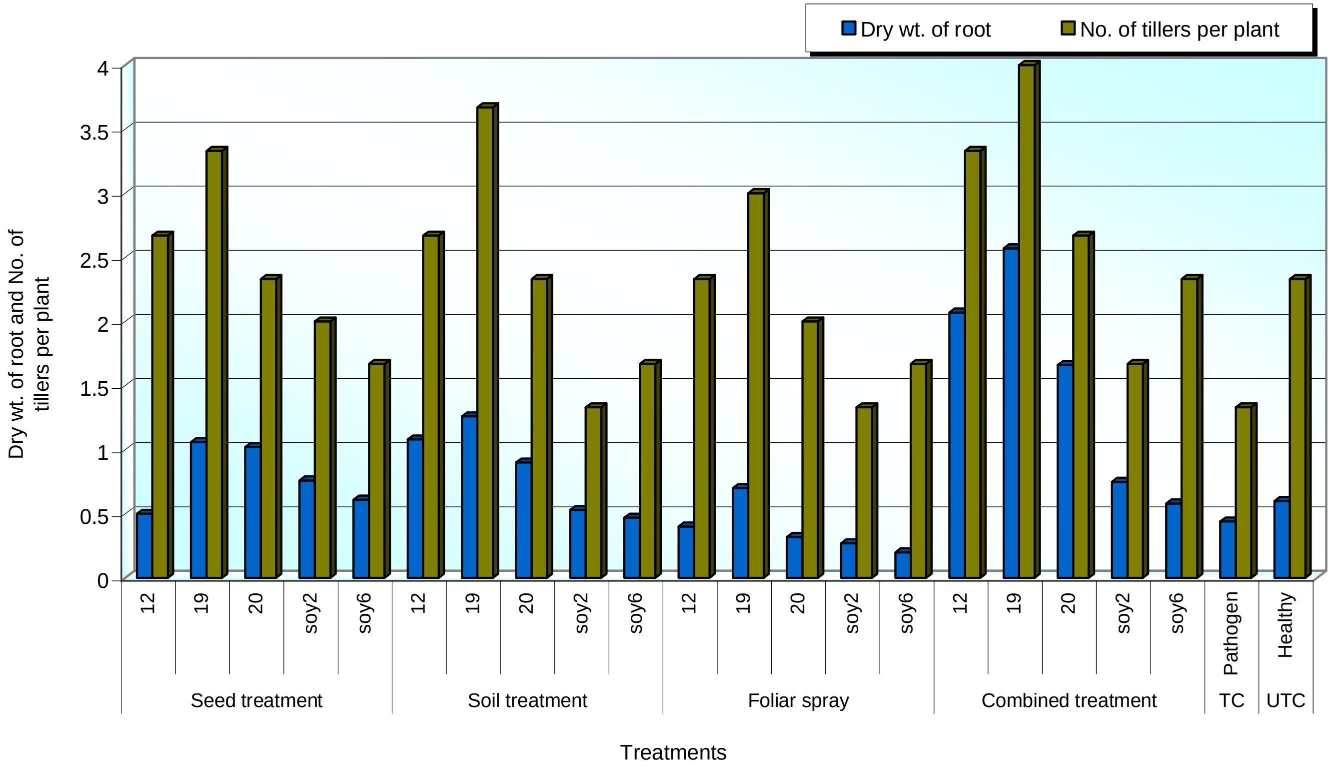


Fig.9b: Effect of different delivery methods of Fluorescent pseudomonad strains on dry weight of root and no. of tillers per plant at different intervals of time

Legend

TC-Treated control, UTC-Untreated control

Fig.9b: Effect of different delivery methods of Fluorescent pseudomonad strains on dry weight of root and no. of tillers per plant at different intervals of time

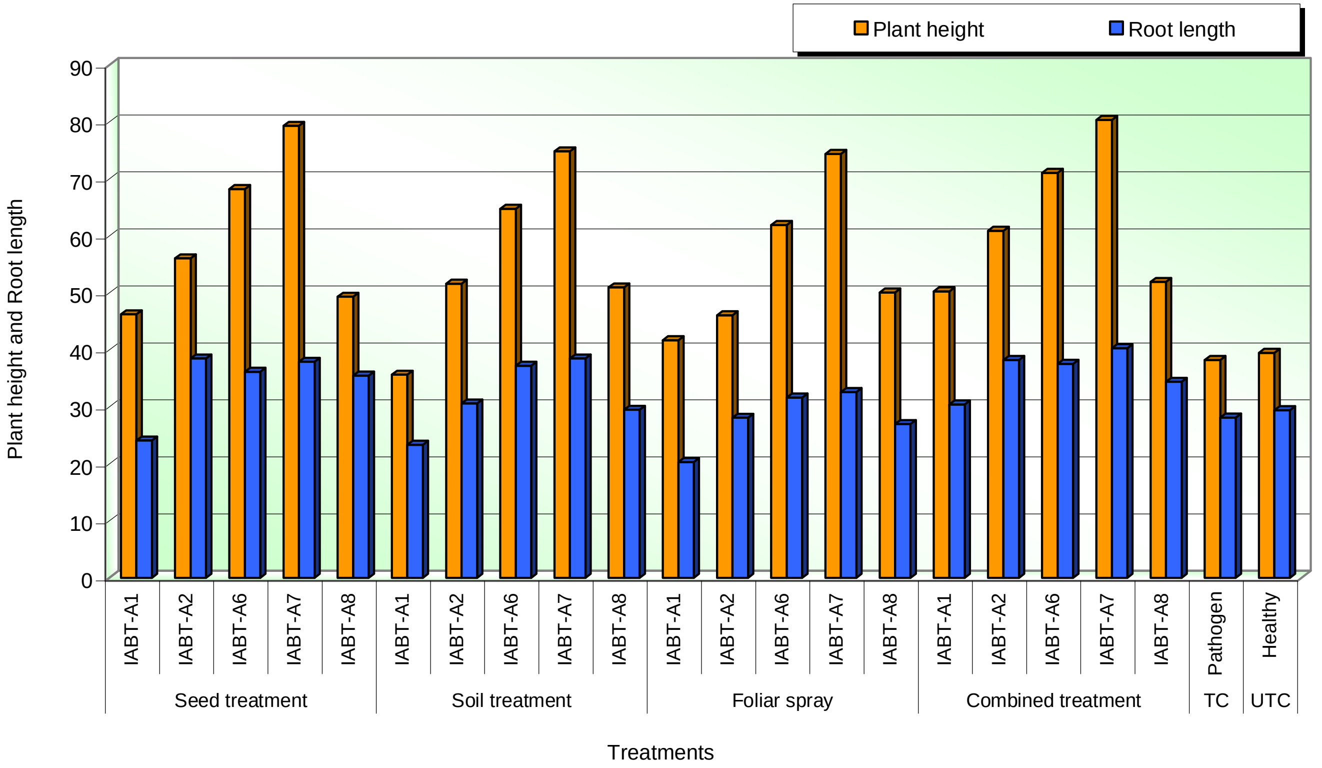


Fig.10a: Effect of different delivery methods of actinomycete isolates on plant height and root length at different intervals of time

Legend
TC-Treated control, UTC-Untreated control

Fig.10a: Effect of different delivery methods of actinomycete isolates on plant height and root length at different intervals of time

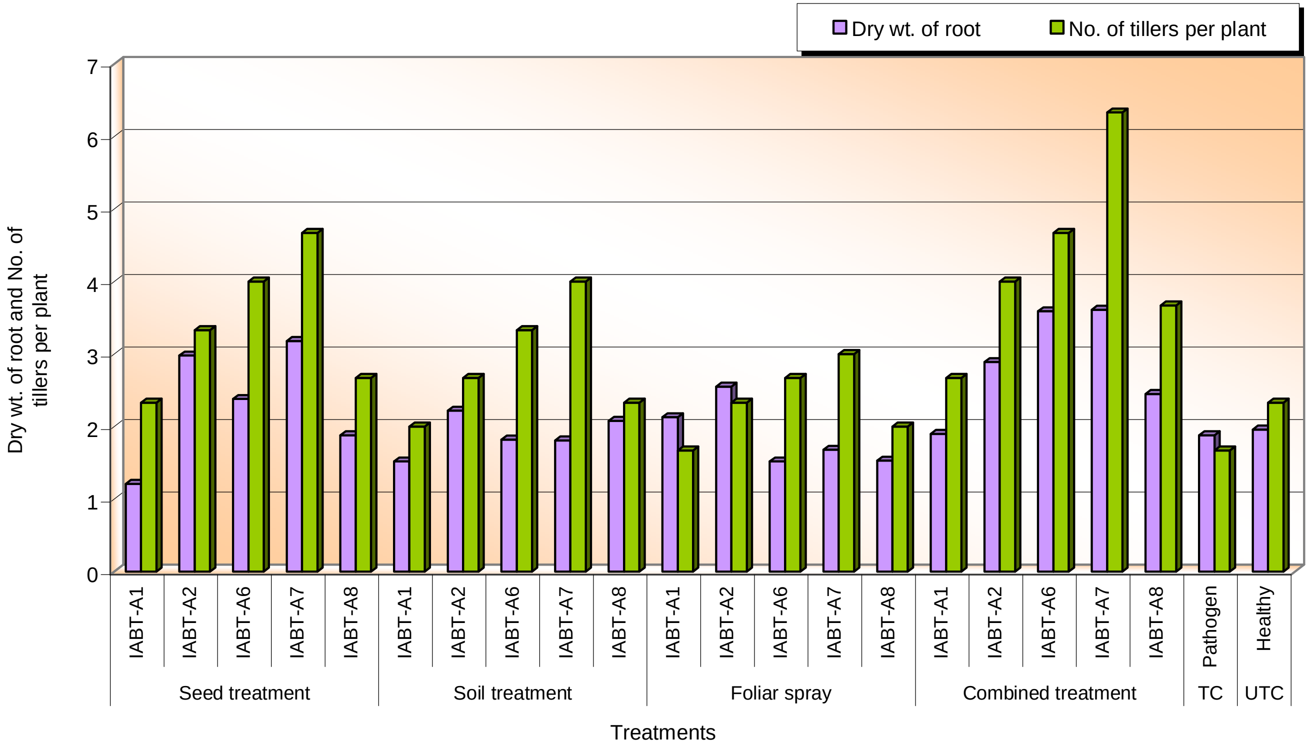


Fig.10b : Effect of different delivery methods of actinomycete isolates on dry weight of root and no. of tillers per plant at different intervals of time

Legend

TC-Treated control, UTC-Untreated control

Fig.10b : Effect of different delivery methods of actinomycete isolates on dry weight of root and no. of tillers per plant at different intervals of time

1-Untreated control (healthy)
2-Foliar spray
3-Soil treatment
4-Seed treatment
5-Combined treatment
6-Treatment control (pathogen)

Plate 13. Effect of actinomycete isolate IABT-A7 on plant growth parameter

Plate 14. Effect of actinomycete isolate IABT-A7 on root growth

Table 15: Impact of potent biocontrol agents on disease and growth parameters

Sl. No.	**Treatment**	**Disease parameters (mean)**		**Growth parameters (mean)**			
		Lesion length (cm)	**No. of dried leaves per plant**	**Plant height (cm)**	**Root length (cm)**	**Dry wt. of root per plant (gm)**	**No. of tillers per plant**
1	Combined treatment* of *Trichoderma harzianum* (1040)	0.69	2.33	N.R.	N.R.	N.R.	3.67
2	Combined treatment* of Fluorescent pseudomonad strain 19	0.22	1.67	61.65	39.50	2.57	4.00
3	Combined treatment* of actinomycete isolate IABT-A7	0.19	1.33	80.37	37.57	3.59	4.67

Combined treatment*- combination of seed treatment+ soil treatment+ foliar spray of biocontrol agent

N.R. - Not Recorded

The integrity of total RNA was examined by electrophoresing the individual RNA samples on denaturing, formaldehyde 1 per cent agarose gel stained with ethidium bromide which clearly showed the presence of two bright bands corresponding to ribosomal *28S rRNA* and *18S rRNA* with a ratio of intensities of ~ 2:1 (Plate 15). The quality of RNA was checked by using NanoDrop ND-1000 spectrophotometer ® (Nano Drop Technologies, Inc. USA) which recorded a ratio of ~ 1.9-2.0 at 260/280 absorbance confirming the purity of RNA for further studies.

4.8.1 Purification of total RNA

To eliminate genomic DNA contamination in RNA preparation about 10 µg of total RNA from each treatment was treated with DNase I enzyme. Again quantity and quality of DNase I enzyme treated RNA was checked using NanoDrop ND-1000 spectrophotometer® which was recorded as a ratio of 2.0 at 260/280 absorbance indicating the purity of RNA.

4.9 Preparation of cDNA

Total RNA of each sample was reverse transcribed by using HighCapacity^{TM} cDNA Reverse Transcription kit (Applied Biosystem) and further resulting sample of cDNA was quantified by using NanoDrop ND-1000 spectrophotometer® in which ratio recorded was 1.78-1.82 at 260/280 absorbance. Quality was checked on 1.5 per cent agarose gel where smear of cDNA was observed.

4.10 Standardization of real-time conditions

4.10.1 Selection of reference genes

Reference genes are used for data normalisation in qRT-PCR, especially when relative quantitation approach is followed. In order to identify the most suitable reference gene in rice, a set of two genes *viz. EF-la, beta-actin,* were selected. The gene expression stability measure (M) was estimated through qRTPCR in all cDNA samples corresponding to healthy plant, pathogen inoculated plant, only bioagent treated plant and pathogen-bioagent inoculated plant. On a model-based variance estimation approach, the M value, which should be < 1.5 for a stably expressed gene, was in the order of magnitude 0.301and 0.306 for *actin* (*AC1*) and *EF-la* respectively. Based on M value *actin* (*AC1*) gene was selected as reference genes for rest of qRT-PCR experiments.

4.11 Expression studies on defence genes and data precision

4.11.1 Induced Systemic Resistance (ISR) related genes

4.11.1.1 Systemic JA-related defence genes in rice

a. Allene Oxide Synthase (*AOS2*) gene

In healthy rice plant *AOS2* gene was not expressed throughout the sampling period from 24hr to 120 hr after sheath inoculation of *R. solani.* The expression was increased along with time interval in seed, combined and only bioagent treatment compared to treated control. However the expression level of the gene was slightly reduced at 72 hrs in the seed, combined and only bioagent treatment. The expression of *AOS2* was significantly upregulated at 120 hr in combined treatment with 1.5 fold increase compared to only pathogen treatment (Table 16, Fig. 11).

b. Jasmonic acid carboxyl methyl transferase (*JMT1*) gene

The gene coding for jasmonic acid carboxyl methyltransferase (*JMT1*), which converts JA to the volatile component MeJA was not expressed in healthy rice plant at all tested time interval. The expression level was gradually increased along with time interval. At 48 hrs the expression level of *JMT1* was gradually increased with 0.32, 0.61 and 0.41 fold in seed, combined and only bioagent treatment but slightly lowered at 72 hrs. The expression of gene was highest at 120 hrs with 1.23 fold change in combined treatment compared to only pathogen treatment (Table17, Fig. 12).

c. JA-inducible Myb transcription factor (*JAMYB*)

The JA-inducible Myb transcription factor, *OsJAMYB* was up regulated in all the treatments except healthy plant at 24 hrs after inoculation. The expression level was gradually decreased along with the time interval in only pathogen treatment. The expression of *OsJAMYB* gradually increased in combined treatment along with time interval and was highest at 120 hrs 3.6 fold change (Table18, Fig. 13).

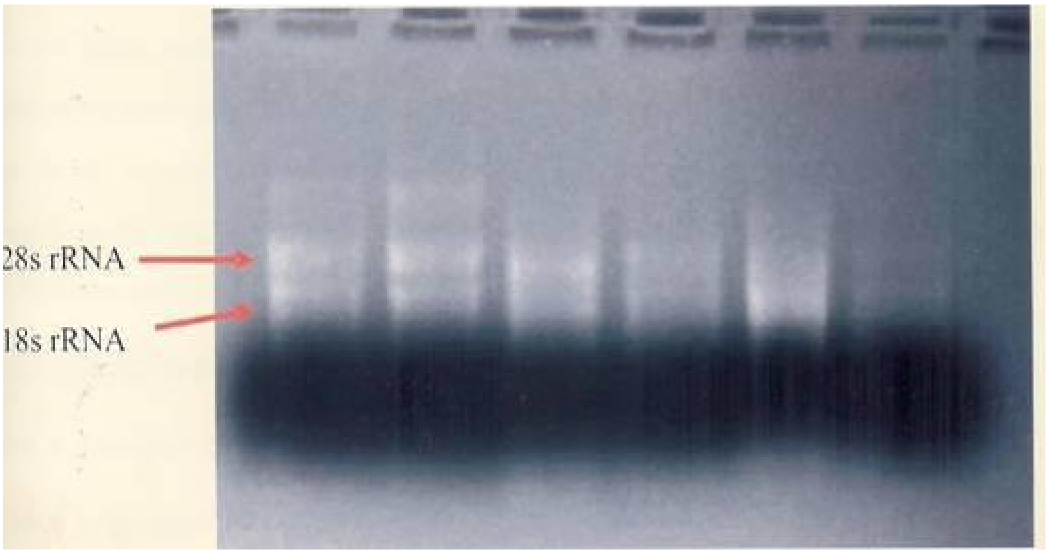


Plate 15. Confirmation of integrity and quality of total RNA on a formaldehyde denaturing agarose gel

Table 16: Relative change in the expression pattern of *Os AOS2* gene at different time interval and in different treatment

Time interval	Treatments	Fold change (Log10)	Cal. t value
24 hr	seed	0.3928	0.2305
	combine	0.4244	0.2154
	O.B	0.2408	0.222
	pathogen	0.0245	0.1973
48 hr	seed	0.4861	0.1414
	combine	0.5448	0.1324
	O.B	0.4590	0.1134
	pathogen	-0.1384	0.1868
72 hr	seed	0.0617	0.1087
	combine	0.3657	0.1291
	O.B	0.3205	0.1257
	pathogen	0.1399	0.1815
120 hr	seed	0.3642	0.2216
	combine	1.5743	0.1637
	O.B	0.7299	0.2846
	pathogen	0.0120	0.1351

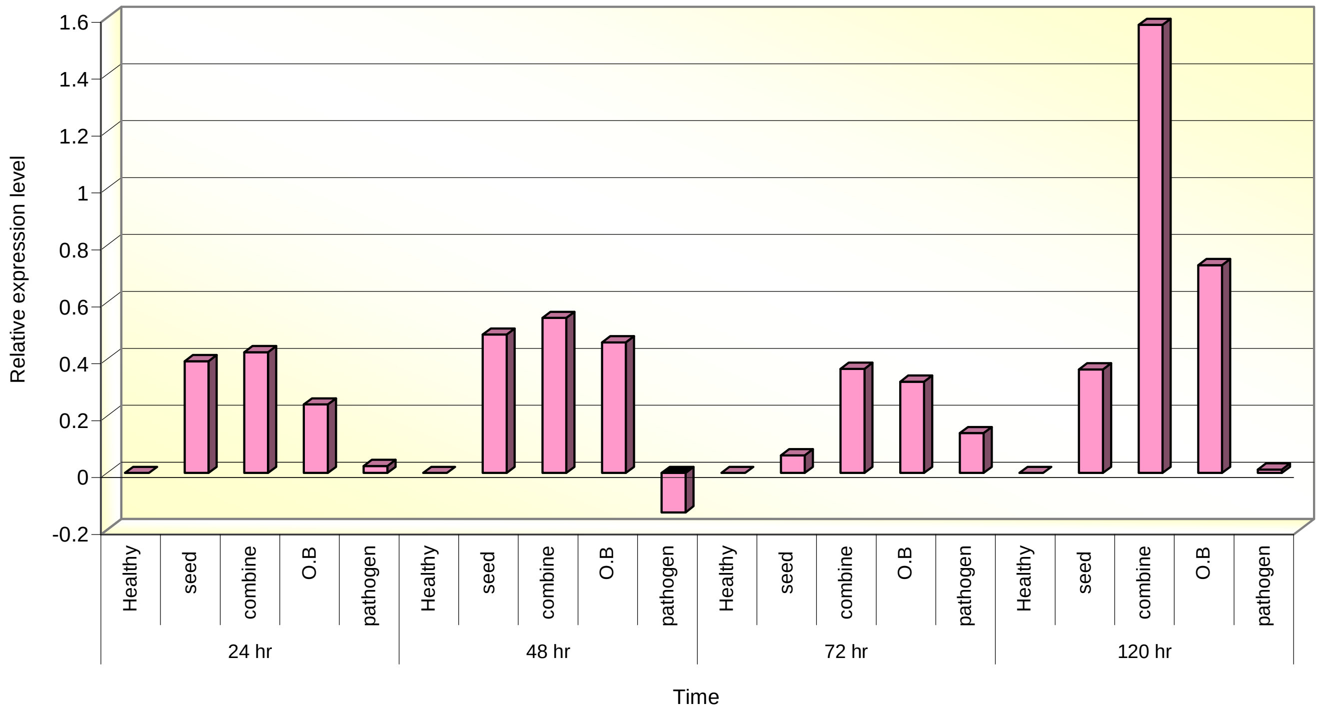


Fig. 11: Analysis of the expression levels of *Os AOS2* gene

Legend
Healthy-Normal plant, Seed-Seed treatment of biogent+pathogen,
Combine-Seed treatment+Soil application+Foliar spraying of bioagent+pathogen,
O.B.-Only bioagent, pathogen-Only pathogen

Fig. 11: Analysis of the expression levels of Os AOS2 gene

4.11.1.2 Systemic Ethylene-related responses

a. Ethylene Responsive Factor (*ERF1*) gene

The ethylene-responsive *OsERF1* gene was expressed after 48hr of challenge inoculation. The expression of gene was down regulated at 24hr in seed, combined and only bioagent treatment. The gene was either not expressed or poorly expressed in healthy and pathogen treatment respectively. The significant up regulation was recorded from 48 hr to 120hr with peak of 2 fold at 72hr in combined treatment. After 72hr of inoculation expression of the gene was declined in all the treatments. In presence of only bioagent treatment up regulation ranged from 1-1.5 fold, whereas, in combined treatment relative up regulation level ranged from 1.4 to 2.1 fold along with time interval (Table19, Fig. 14).

b. Ethylene Insensitive2 (*EIN2*) gene

OsEIN2 is a central signal transducer in the ET signaling pathway. The expression and up regulation of gene was very poor from 24hr to 72hr of inoculation in all the treatments. At 24 hrs of inoculation there was slight down regulation of *OsEIN2* in seed and only bioagent treatment. However, the gene expression was highly up regulated at 120hr in seed and combined treatments. In combined treatment gene expression was maximum with 2.2 fold at 120hr over only pathogen treatment (Table 20, Fig. 15).

4.11.2 Systemic Acquired Resistance (SAR) related gene

4.11.2.1 Systemic SA-related defence gene in rice

a. Pathogenesis related 1b (*PR1b*) gene

The gene started expressing immediately after challenge inoculation of pathogen. The gene expression was observed even in seed and combined treatments in addition to only pathogen treatment. The expression of the gene was drastically declined in pathogen-bioagent interaction treatments contrary to high expression in only pathogen treatment from 24 to 72 hrs. The relative expression of the gene was highest in pathogen treatment at 48 and 72 hrs interval sample drawn with 12-10 fold. The expression of gene was down regulated after 72hrs of infection with least expression at 120hr with 2 fold change (Table 21, Fig.16).

b. Non pathogenesis related (*NPR1*) gene

The expression of gene *NPR1* was up regulated immediatly after the pathogen inoculation in seed, combined and only bioagent treatment in all the time intervals. Though the gene expression was up regulated in only pathogen treatement at 48 and 72 hrs, the expression was down regulated at 120hrs. The expression of *NPR1* was highly up regulated in combined treatment from 24 hrs to 120 hrs with maximum expression at 120 hrs with 1.6 fold change (Table 22, Fig.17).

On the other hand some gene related to Systemic ET-related responses as 1-Aminocyclopropane-1-carboxylate synthase (ACS) gene and 1-Aminocyclopropane-1-carboxylate oxidase (ACO7) gene and related to Systemic SA-related defence gene in rice as Isochorismate synthase (ICS) gene and Phenylalanine ammonia-lyase (PAL) gene did not shown any Ct value which indicated that no amplification were occurred which inference as these genes were not expressed in any treatment.

Table 17: Relative change in the expression pattern of *Os JMT* gene at different time interval and in different treatment

Time interval	Treatments	Fold change (Log10)	Cal. t value
24 hr	seed	0.1490	0.9264
	combine	0.4605	0.9012
	O.B	-0.1475	0.8882
	pathogen	-0.1324	0.8944
48 hr	seed	0.3221	0.7945
	combine	0.6186	0.7766
	O.B	0.4665	0.7112
	pathogen	0.0135	0.7095
72 hr	seed	0.0737	0.8178
	combine	0.1610	0.7088
	O.B	0.0135	0.7985
	pathogen	0.0842	0.7461
120 hr	seed	1.0626	0.7301
	combine	1.2342	0.8183
	O.B	0.9617	0.8362
	pathogen	0.0000	0.8026

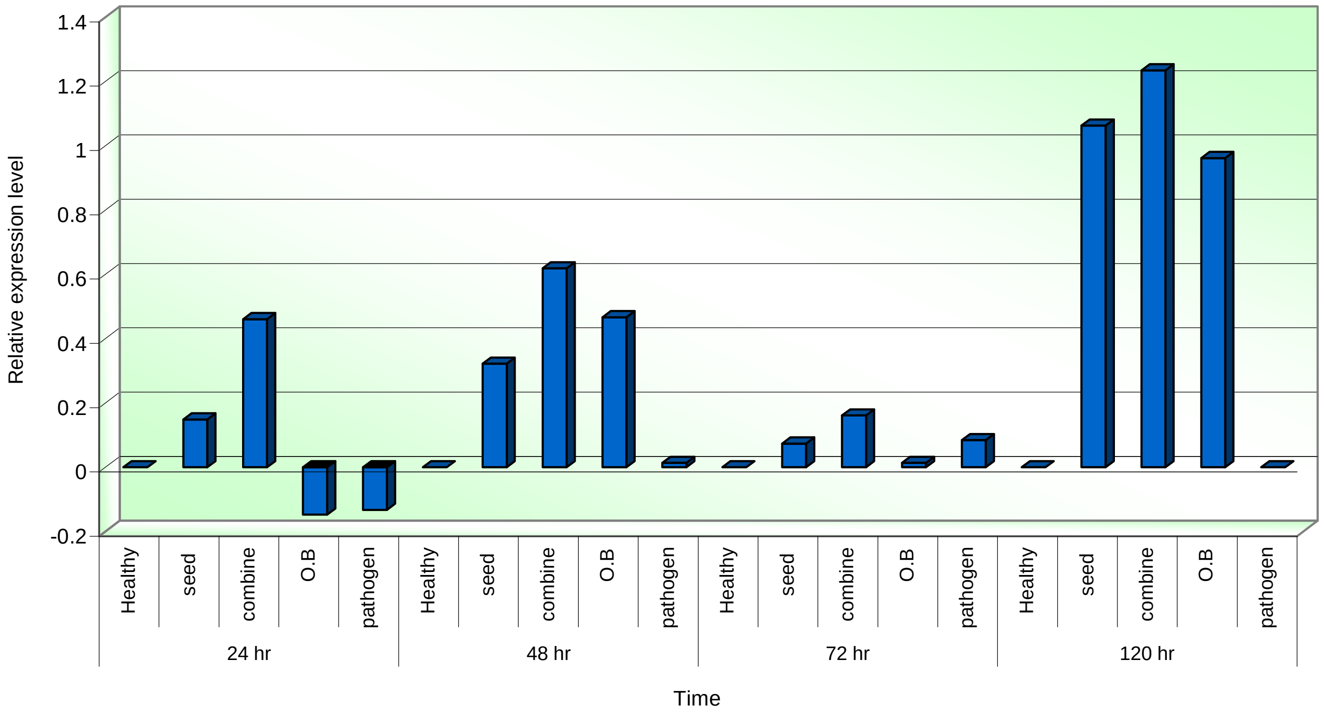


Fig. 12: Analysis of the expression levels of *Os JMT* gene

Legend
Healthy-Normal plant, Seed-Seed treatment of biogent+pathogen,
Combine-Seed treatment+Soil application+Foliar spraying of bioagent+pathogen,
O.B.-Only bioagent, pathogen-Only pathogen

Fig. 12: Analysis of the expression levels of Os JMT gene

Table 18: Relative change in the expression pattern of *Os JAMYB* gene at different time interval and in different treatment

Time interval	Treatments	Fold change (Log10)	Cal. t value
24 hr	seed	0.7420	0.4288
	combine	1.2884	0.4897
	O.B	1.1183	0.4027
	pathogen	1.0566	0.5573
48 hr	seed	1.1574	0.5231
	combine	1.8392	0.5668
	O.B	2.3856	0.4993
	pathogen	0.3762	0.4164
72 hr	seed	1.9220	0.5226
	combine	2.4564	0.5349
	O.B	0.8398	0.5316
	pathogen	0.0526	0.5017
120 hr	seed	2.8131	0.4312
	combine	3.6018	0.4858
	O.B	2.3450	0.4847
	pathogen	0.1681	0.4969

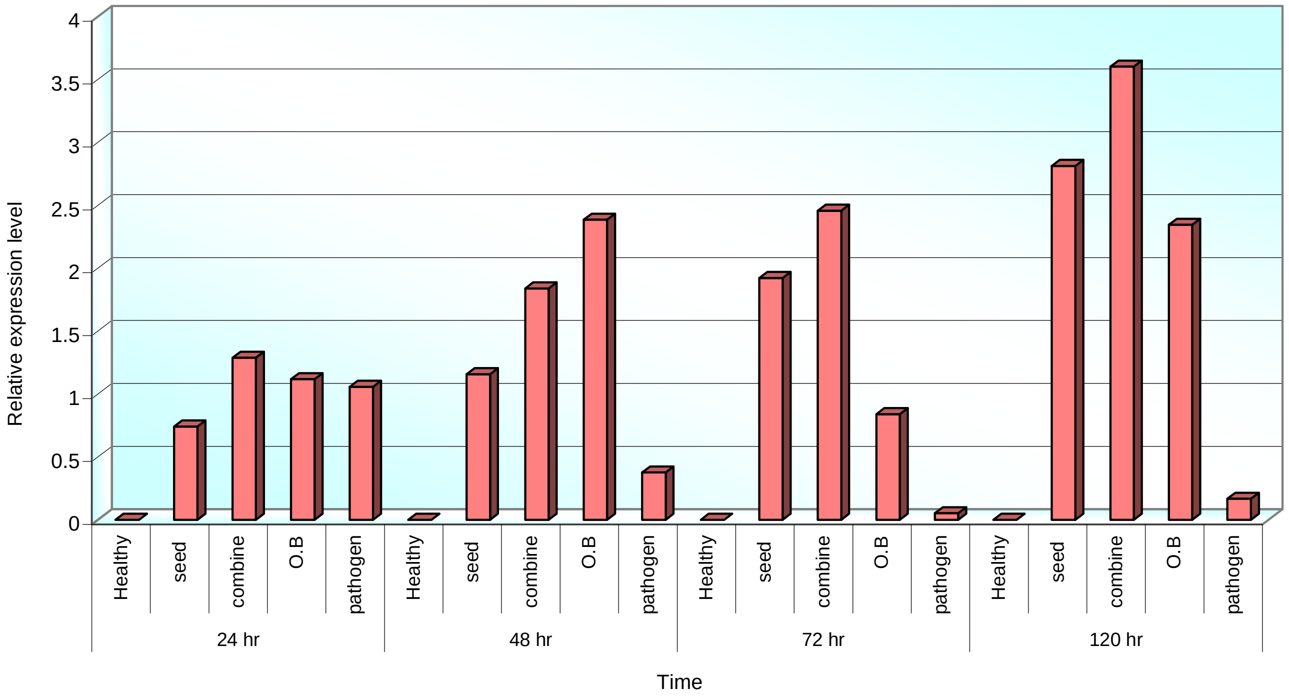


Fig. 13: Analysis of the expression levels of *Os JAMYB* gene

Legend
Healthy-Normal plant, Seed-Seed treatment of biogent+pathogen,
Combine-Seed treatment+Soil application+Foliar spraying of bioagent+pathogen,
O.B.-Only bioagent, pathogen-Only pathogen

Fig. 13: Analysis of the expression levels of Os JAMYB gene

Table 19: Relative change in the expression pattern of *Os ERF1* gene at different time interval and in different treatment

Time interval	Treatments	Fold change (Log10)	Cal. t value
24 hr	seed	-1.3651	0.2945
	combine	-1.1348	0.3137
	O.B	-1.3245	0.3294
	pathogen	0.0000	0.2011
48 hr	seed	0.7104	0.1142
	combine	1.5487	0.1263
	O.B	1.1514	0.1547
	pathogen	0.1535	0.1162
72 hr	seed	1.5698	0.2151
	combine	2.0996	0.2248
	O.B	1.6165	0.2596
	pathogen	0.3010	0.2161
120 hr	seed	1.0024	0.2198
	combine	1.7444	0.2018
	O.B	0.8850	0.1608
	pathogen	0.0000	0.2035

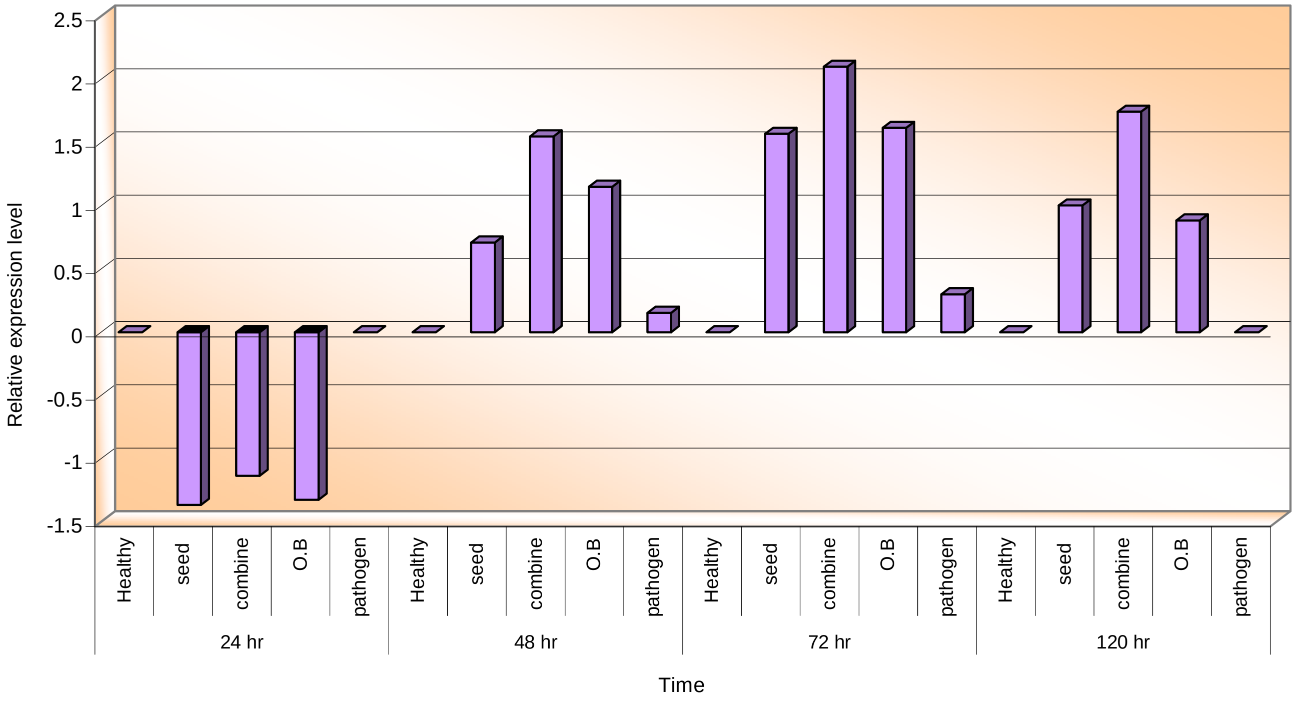


Fig. 14: Analysis of the expression levels of *Os ERF1* gene

Legend
Healthy-Normal plant, Seed-Seed treatment of biogent+pathogen,
Combine-Seed treatment+Soil application+Foliar spraying of bioagent+pathogen,
O.B.-Only bioagent, pathogen-Only pathogen

Fig. 14: Analysis of the expression levels of Os ERF1 gene

Table 20: Relative change in the expression pattern of *Os EIN2* gene at different time interval and in different treatment

Time interval	Treatments	Fold change (Log10)	Cal. t value
24 hr	seed	-0.0842	0.1168
	combine	-0.0105	0.1285
	O.B	-0.1640	0.1326
	pathogen	-0.0436	0.1279
48 hr	seed	-0.0662	0.2248
	combine	-0.0541	0.2313
	O.B	-0.0135	0.2787
	pathogen	0.0361	0.2204
72 hr	seed	-0.0466	0.23
	combine	0.3356	0.2696
	O.B	0.0406	0.2532
	pathogen	0.0165	0.1106
120 hr	seed	1.5743	0.1258
	combine	2.2050	0.0378
	O.B	0.7239	0.1142
	pathogen	0.0210	0.1292

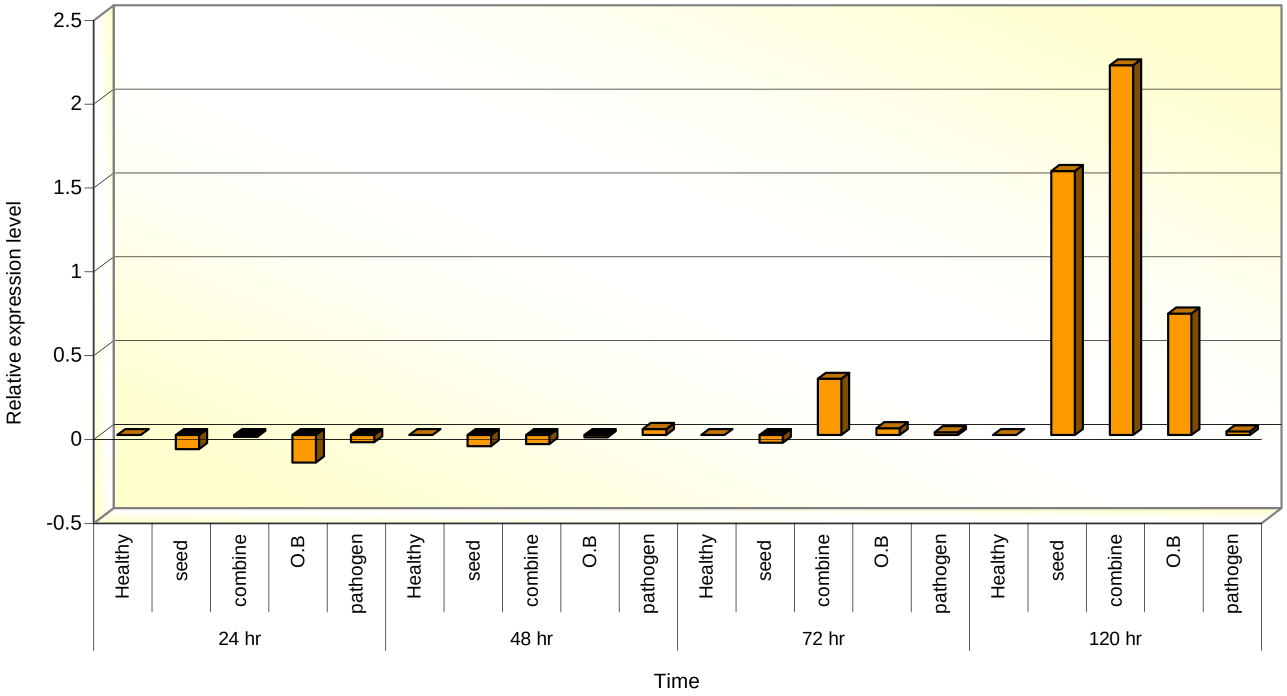


Fig. 15: Analysis of the expression levels of *Os EIN2* gene

Legend

Healthy-Normal plant, Seed-Seed treatment of biogent+pathogen,
Combine-Seed treatment+Soil application+Foliar spraying of bioagent+pathogen,
O.B.-Only bioagent, pathogen-Only pathogen

Fig. 15: Analysis of the expression levels of Os EIN2 gene

Table 21: Relative change in the expression pattern of *OsPR1b* gene at different time interval and in different treatment

Time interval	Treatments	Fold change (Log10)	Cal. t value
24 hr	seed	5.1114	0.1342
	combine	10.6504	0.2848
	O.B	-0.2498	0.2908
	pathogen	2.7559	0.304
48 hr	seed	1.8122	0.1421
	combine	1.9612	0.2201
	O.B	-0.4304	0.1236
	pathogen	12.0201	0.1116
72 hr	seed	1.3591	0.1235
	combine	1.9566	0.1226
	O.B	-0.5584	0.1671
	pathogen	10.5074	0.2198
120 hr	seed	1.8648	0.1889
	combine	1.2643	0.1695
	O.B	-0.2077	0.2556
	pathogen	2.4564	0.3783

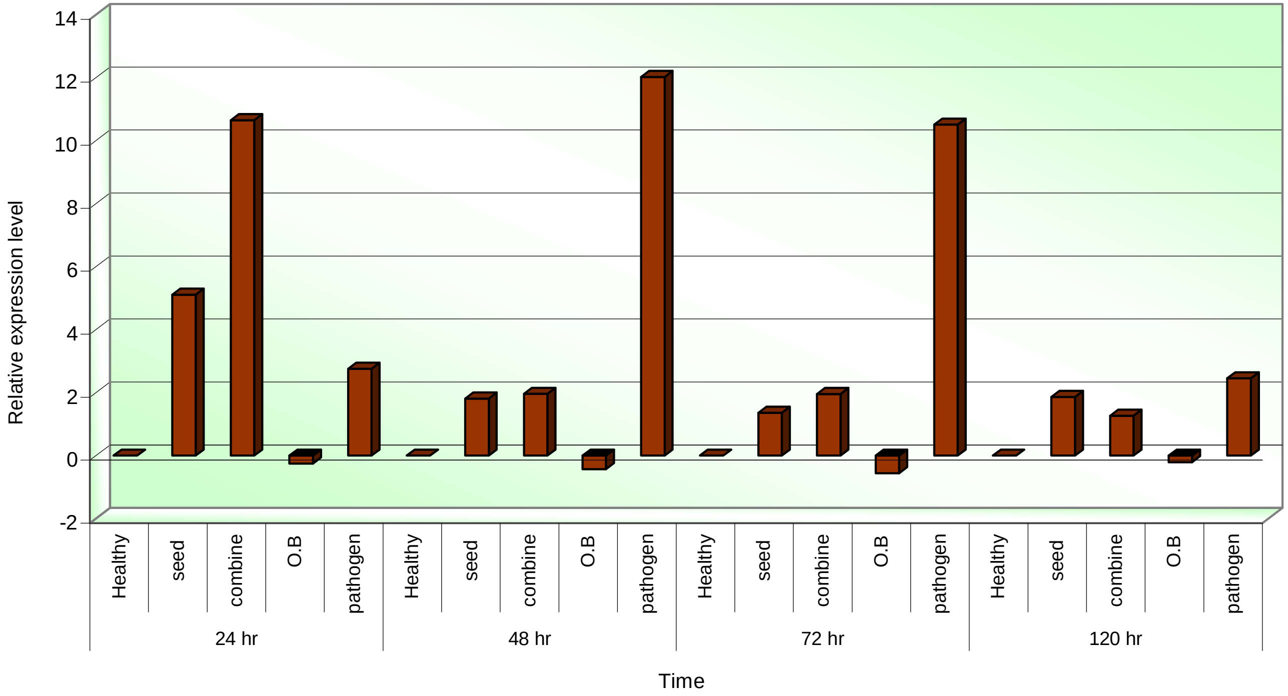


Fig. 16: Analysis of the expression levels of *Os PR1b* gene

Legend
Healthy-Normal plant, Seed-Seed treatment of biogent+pathogen,
Combine-Seed treatment+Soil application+Foliar spraying of bioagent+pathogen,
O.B.-Only bioagent, pathogen-Only pathogen

Fig. 16: Analysis of the expression levels of Os PR1b gene

Table 22: Relative change in the expression pattern of *Os NPR1* gene at different time interval and in different treatment

Time interval	Treatments	Fold change (Log10)	Cal. t value
24 hr	seed	1.2236	0.137
	combine	0.1053	0.1753
	O.B	0.3446	0.1657
	pathogen	0.0135	0.2154
48 hr	seed	1.0385	0.1418
	combine	0.6893	0.1214
	O.B	0.9918	0.1488
	pathogen	0.2588	0.1148
72 hr	seed	1.3395	0.126
	combine	1.5999	0.1782
	O.B	0.4846	0.1366
	pathogen	1.2101	0.1487
120 hr	seed	1.1634	0.9447
	combine	1.6887	0.9943
	O.B	0.7480	0.9496
	pathogen	-0.3085	0.1096

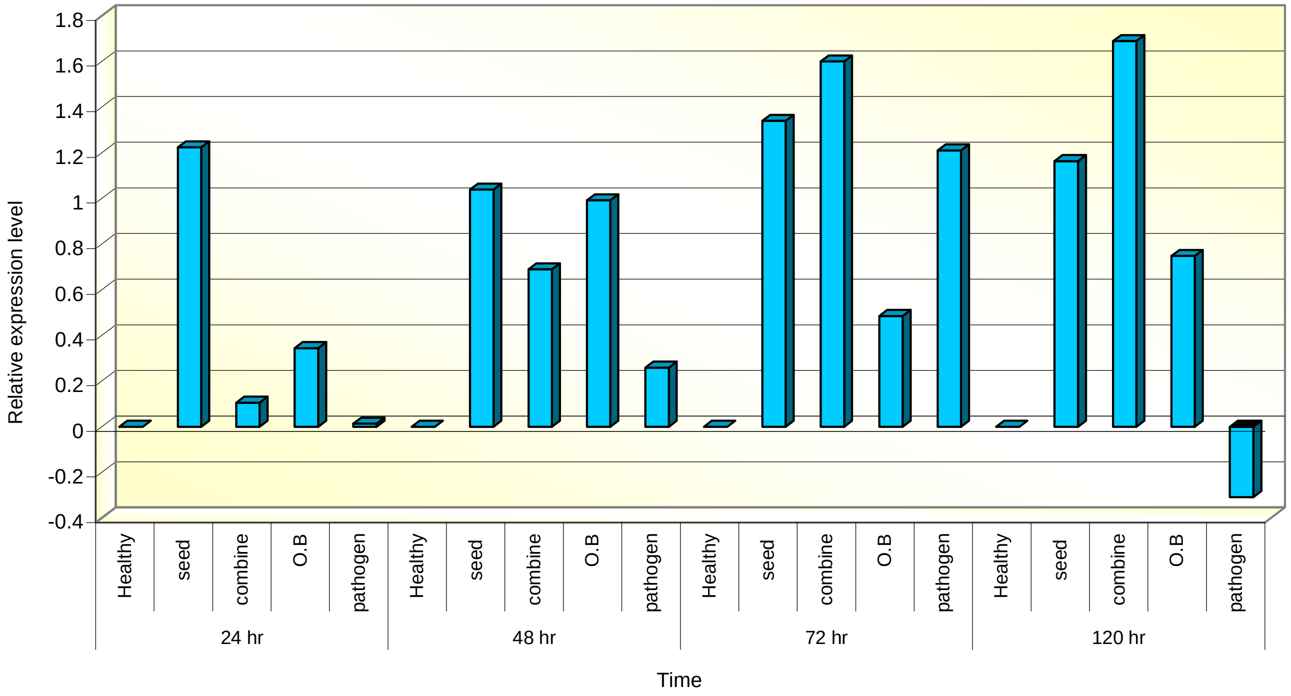


Fig. 17: Analysis of the expression levels of *Os NPR1* gene

Legend
Healthy-Normal plant, Seed-Seed treatment of biogent+pathogen,
Combine-Seed treatment+Soil application+Foliar spraying of bioagent+pathogen,
O.B.-Only bioagent, pathogen-Only pathogen

Fig. 17: Analysis of the expression levels of Os NPR1 gene

DISCUSSION

The results obtained under the present investigation on identification of potential biocontrol agent against *R. solani* and their molecular interaction in host is discussed hereunder.

5.1 Selective isolation of actinomycetes and Molecular characterization and identification of potent actinomycete isolates at genus level

Actinomycete isolates were enumerated from rhizosphere soil of healthy rice plants. For the isolation of actinomycete population, media with high C: N ratio like starch casein agar (SCA) is recommended, since other bacteria cannot thrive on high molecular weight and resistant polymers like starch or casein (Grey and Williams, 1971). Hence, in this study starch casein agar media was preferred for specific isolation of actinomycetes. Antibiotics, cycloheximide (100µg/ml) and streptomycin (50µg/ml) was added to the media (Tachowisan *et al.*, 2003) to avoid the growth of fungus and bacteria.

On the basis of characteristic colony morphology like compact sporulation, chalk like dry colonies of different colours ranging from white, grey, reddish or yellowish *etc.*, eleven colonies were putatively selected (Arifuzaman *et al.*, 2010; Lo *et al.*,2002). Further they were identified based on 16S rDNA and ARDRA and confirmed as actinobacteria.

5.1.1 PCR based identification of actinomycetes

The isolates were subjected to 16S rDNA test of prokaryotes (16S rDNA region) and ITS test (18S rDNA) of eukaryotes (18S rDNA region). PCR amplification was shown that all eleven isolates got amplification in 16S rDNA test with amplicon size approximately 1.5kb (Plate 3) but not amplified in ITS test which proved that all isolated cultures were belonging to prokaryotes at genetic level (Anderson and Wellington, 2001).

5.1.2 Identification of actinomycete isolates at genus level

PCR-based methods have provided a rapid and accurate way to identify bacteria (Wilson *et al.*, 1998; Laurent *et al.*, 1999). In particular, amplified rDNA restriction analysis (ARDRA) has proved to be very useful (Harvey *et al.*, 2001; Alves *et al.*, 2002) for identification actinomycetes. The detection and identification of actinomycetes genera is essential. ARDRA has been shown to be useful in differentiating between bacterial species within a genus, for example, *Actinomadura, Gordonia, Nocardia, Rhodococcus, Saccharomonospora, Saccharopolyspora, Streptomyces and Tsukamurella* (Cook *et al.*, 2003). In the present research, based on ARDRA analysis three potent isolates were identified as *Actinopolymorpha* spp. (IABT-A1, IABT-A6 and IABT-A7) and remaining were identified as *Streptomyces* spp. (Plate 4).

5.2 Inoculation of pathogen

An effective inoculation method is a critical component of an accurate disease assay for quantifying levels of sheath blight resistance among rice cultivars. The type of inoculum used in an inoculation method is a major determinant of infection efficiency. Several previous studies used toothpicks colonized with *R. solani* (Rodrigues *et al.,* 2003; Zou *et al.,* 2000), sclerotia (Singh *et al.,* 2001) and infected rice grain–hull mixtures (Pan *et al.,* 1999; Willocquet *et al.*, 2000). The time required for these inoculum sources to begin active growth in order to initiate infection can vary widely depending on environmental and pathogen factors.

Singh *et al.* (2001) also reported that infection was most rapid when young mycelium was used as inoculum and slowest with mature sclerotia. These pathogen factors led to additional variation in the infection process and the subsequent disease development. For this reason, only actively growing sources of *R. solani* inoculum were analyzed in this study. Three different methods of pathogen inoculation were used. First is infection to sheath as agar block and liquid cultured mycelia ball and third is soil treatment. Agar blocks produced the longest lesions on rice sheaths 7 days after inoculation; in fact, more than twice that produced by colonized mycelial balls or mycelial suspensions. Liquid cultured mycelia ball produced lesions on rice sheaths 21 days after inoculation and soil treatment produced lesions on rice sheaths 30 days after inoculation. These results were significant and consistent in repeated experiments. One possible reason for this is that agar blocks provide greater fungal biomass and moisture than the other two types of inocula.

5.3 Screening of biocontrol agents against *R. solani* under *in vitro* condition

Biological control through the use of antagonistic microorganisms is a potential, non chemical means of controlling plant diseases by reducing inoculum levels of pathogens. Such a management would help in preventing the pollution and also health hazards. In the present investigation, the antagonistic effect of different isolates of bioagents viz., *Trichoderma spp*. (13 isolates), Fluorescent pseudomonad (15 strains), Methylobacterial strains (11 strains) and actinomycetes (11 isolates) were assessed against *R. solani* by dual culture technique.

All the trichoderma isolates significantly reduced the growth of *R. solani* compared to control. Among different Trichoderma isolates assayed for the control of *R. solani T. harzianum* (1034) and *T. virens* (1010) were significantly effective with 69.33 per cent and 73.10 per cent inhibition of mycelial growth of VC Farm Mandya and Mandya HRL isolates respectively. Earlier workers reported about the potentiality of trichoderma isolates against *R. solani* in tomato (Montealegre *et al*., 2010). *Trichoderma* use different mechanisms for the control of phytopathogens which include mycoparasitism,competition,secretion of antibiotics and fungal cell wall degrading enzymes (Harman *et al*.,2004).Several researchers reported the inhibitory effect of volatile and non volatile substances produced by *Trichoderma* spp., on several soil borne plant pathogens (Amin *et al*., 2010). The inhibitory activity of *Trichoderma* spp. might be due to diffusible metabolites secreted by them against the pathogen for inhibition of mycelial growth.

Among fifteen F. pseudomonad strains screened against *R. solani* under *in vitro* condition, five strains were found to be potent antagonists. The per cent inhibition varied from 63.70-100 per cent. However, F. pseudomonad strain 19 showed 100 per cent inhibition against both Mandya HRL and VC Farrm Mandya isolates of *R. solani* followed by strains 6, 12, 20, 30, 22, 25, soy6 which showed 90-100 per cent inhibition against VC Farm Mandya and Mandya HRL isolates. Due to the presence of iron chelating ability, a siderophore producing bacterium inhibit harmful microorganisms by competing for iron and thus reduces the levels of freely available ferric ions (Sayyed *et al*.,2005). Furthermore, chemically, siderophores are phenolic compounds, which are antimicrobial in nature and may be responsible for antifungal activity of the test pathogen. The mycoparasitic potential of *Pseudomonas* spp. is well documented (Keel and Defago, 1997). Thus, this phenomenon has often been used as means for *in vitro* screening of biocontrol agents (Crowe *et al.* 2001). Various researchers also demonstrated the role of rhizobacteria in the inhibition of *R. solani* and the mechanisms with which they bring about the beneficial effect. The mechanism behind inhibition of *R. solani* from Fluorescent pseudomonad may be attributed to antibiotics and siderophore production (Rini *et al.* 2007; Reddy *et al.* 2010)

Twelve *Methylobacterial* strains were tested for biocontrol potential against *Rhizoctonia solani*. The isolates 38U and 42U significantly reduced the linear mycelial growth of pathogen and effective inhibition over the other strains. The earlier reports suggest the effect of volatile antibiotics on the mycelial growth of fungal pathogen (Poorniammal *et al*., 2009).

The effect of actinomycetes on *Rhizoctonia solani* was quite eye-catching. Ten isolates out of eleven exhibited 90-100 per cent inhibition. The highest inhibition (100 per cent) was shown by IABT-A2, IABT-A3, IABT-A7, and IABT-A8 against two different isolate of *Rhizoctonia solani.* The growth rate of mycelium was reduced compared to control may be due to siderophore production (Hassanin *et al*., 2007). The antifungal activity exhibited by actinomycete isolates might be due to secretion of hydrolytic enzymes such as chitinase,β-1,3 glucanase, chitosanase and proteases (Islam *et al*., 2009) which degrade the fungal cell wall or the secretion of antifungal compounds (Khamna *et al*., 2009).

It has been found that the *Rhizoctonia solani* can be controlled by the antifungal activity of *Trichoderma* isolates*,* pseudomonas strains, *Methylobacterial* strains and actinomycete isolates. Actinomycete isolate and pseudomonas strains performed well and gave higher inhibition followed by *Trichoderma* isolates against different isolates of *R. solani* and they were further screened under *in vivo* condition*.*

5.4 Evaluation of bioagents against *R. solani* under glasshouse condition

The isolates which were potent under *in vitro viz.*,trichoderma isolates as *T. virens* (1002), *T. virens* (1010), *T. harzianum* (1034), *T. harzianum* (1036) and *T. harzianum* (1040), pseudomonas strains as 12,19,20, soy2 and soy6, actinomycete isolate as IABT-A1,2,6,7 and 8 under in vitro condition were evaluated in greenhouse conditions. To know the efficacy of bioagents under *in vivo* condition different application methods *viz.*, seed treatment, soil inoculation, foliar spraying and combined treatment.

Amongst different methods of application, the combined application method was found to be the most efficient method followed by seed treatment. This method improved plant growth, growth components and less sheath blight infection. Combined application of bioagents on disease control parameters namely lesion length and number of dried leaves were assessed. Further, the impact of bioagents increased the plant height, number of tillers, root length, root biomass when compared to the pathogen inoculated control (Table 13 and 14) was assessed. The bioagents significantly reduced disease compared to untreated check. However, among bioagents *Trichoderma spp.* could not significantly reduce the lesion length and number of dried leaves as compared to pseudomonas and actinomycete isolates. Hence, growth parameters *viz.* plant height, shoot and root weight, number of tillers and dry weight of roots were recorded for pseudomonad and actinomycete isolates treatments.

Under *in vivo* condition effect of Fluorescent pseudomonad on sheath blight disease parameter was recorded. The combined treatment of Fluorescent pseudomonad strain 19 showed the lowest lesion length which was 0.11 cm at 40 DAS and 0.32 cm at 60 DAS with mean 0.22 cm and number of dried leaves was 1.67 leaves/plant. A combined application of bacteria suspension isolates was most effective method for control of rice disease in the field. Possibly both rhizosphere and phyllosphere population of Fluorescent pseudomonad helped to control disease. Both direct inhibition of pathogen and systemically induced resistance in the rice plants could be involved in control (Kazempour, 2004).Combined application of strain 19 resulted in growth promotion compared to check. It increased plant height as (62.18 cm,) number of tillers as (4/plant), root length as (41.71 cm), dried weight of root as (2.57g/plant). The increase in shoot and root length especially in combined treatment may be related to the action of cellulose and pectinases on host cell walls which would decrease the level of lignin cell wall- bound phenolic compounds (Anitha and Das, 2011). The biocontrol agent belong to the bacteria genus *Pseudomonas* utilized in low-input sustainable agricultural applications, such as biocontrol, on account of their ability to synthesize secondary metabolites with antibiotic properties. These secondary metabolites include 2,4 diacetylphloroglucinol (DAPG), phenazine (Phz), pyrrolnitrin, oomycin A, viscosinamide, pyoluteorin and hydrogen cyanide (HCN). Among these secondary metabolites, DAPG has received the particular attention because of its production by a wide range of pseudomonads used for the biological control of root diseases (Lanwongsa, 2010).

And in the present study, spectacular performance of the combined treatment of actinomycete under *in vivo* condition explained by better establishment of the bioagent against the targeted test pathogen. The combined treatment of IABT-A7 showed the lowest lesion length which was 0.11 cm at 40 DAS and 0.29 cm at 60 DAS with mean 0.19 cm and number of dried leaves was 1.33 leaves/plant. Specifically, colonization or the biosynthesis of antibiotics and other secondary metabolites can prevent pathogen invasion and establishment (Poorni *et. al.*, 2011). Further, the growth parameters were observed as highest plant height in IABT-A7 as 80.89 cm/plant, number of tillers as 6.33 per plant root length as 40.30 cm, dried weight of root as 3.18g/plant. Direct promotion of plant growth by PGPR occurs when the plant is supplied with a compound that is synthesized by the bacteria, or when PGPR otherwise facilitates plant uptake of soil nutrients. Possibilities include nitrogen fixation, siderophore synthesis, phytohormone synthesis, and solubilization of minerals to make them available for plant uptake and use (Srividya *et. al.,* 2012).

The present investigations further revealed the antagonistic nature of actinomycet isolate (IABT-A7) against *R. solani* was more efficient than other actinomycete isolates and pseudomonas strains in case of reduction in disease parameter and increase in plant growth parameter shown in Table 15. Hence to observe more efficiency of IABT-A7 at molecular level it was selected for further molecular study.

This study also revealed that the reason why seed treatment is better than soil application of antagonist is in soil applied with bioagents, the bioagents at first have to multiply in the introduced environment and then it has to move near the root region to prevent the entry of pathogen. In seed pelleting treatment, the antagonistic organism readily multiplies on seed surface, which in turn prevents the entry of the pathogen (Raguchander *et al.,* 1998).

Significant increases in plant growth parameters in the present study may be attributed to the production of plant growth regulators such as auxins, gibberellins, cytokinins and ethylene (Frankenberger and Arshad, 1995). It has often been inferred that rhizobacterially produced auxins are responsible for growth promotion. Indole acetic acid promotes ethylene production by stimulating the enzyme in the ethylene biosynthetic pathway (Suneesh, 2004).

To ensure consistent performance of bioagents on growth promotion and minimize the disease incidence the application methods are often considered being an important aspect. Hence, different delivery methods were evaluated. With different application methods of pseudomonad strain and actinomycete isolates, the effects on growth promotion and disease reduction varied. In general, less plant height of rice was observed in case of challenge inoculation with the pathogen at all periods of observation. It is evident from the results that there was significantly improved growth and low lesion length of disease incidence due to different methods of inoculation of pseudomonad strains and actinomycetes at all intervals of time. This may be due to the production of copious amounts of phytohormones such as IAA and GA and mineral phosphate solubilization ability of the strain.

5.5 Total RNA isolation

A critical step in the successful implementation of RNA based techniques for molecular biology and functional genomic experiments (e.g. SSH and Real time PCR) is the routine isolation of high quality RNA (Iandolino *et al.*, 2004).

RNA isolation presents more challenges than DNA isolation because of the susceptibility of RNA to degradation by ribonucleases (RNases). RNases are very stable and active enzymes which do not generally require cofactors to function. Since, only minute amounts of RNases are sufficient to destroy RNA; extreme caution is needed with an effective RNA isolation procedure.

In the present study to avoid RNA degradation, all the glassware, mortar and pestle, microcentrifuge tubes and microtips were treated with autoclaved DEPC treated water. DEPC (Diethyl pyrocarbonate) alkylates the histidine molecules present in the active site of RNase enzyme thus inhibiting its activity. DEPC treated water was autoclaved in order to remove traces of DEPC that might otherwise modify purine residues in RNA by carboxymethylation and thus affecting its translation. Also gloves were worn throughout the experiment, to avoid RNA degradation from RNase that is present in the working hands.

Another problem in isolating high quality RNA is the interference with aqueous metabolites like phenols and polysaccharides which accumulates in tissues and may be purified along with nucleic acids (Loomiz, 1974; Stokes *et al.*, 1990). In present investigation young, tender leaves from different treated plants viz. combined treatment of actinomycete (IABT-A7), seed treatment of actinomycete (IABT-A7), only bioagent (actinomycete IABT-A7) treated, only pathogen (*R. solani*) and absolute plants (healthy) at different time intervals like 24hr, 48hr, 72hr, 120hr after pathogen inoculation to sheath were used for isolation of total RNA, since nucleic acid yields from young tissues are often higher than old tissue. This is because young tissue generally contains actively dividing cells in addition it may have fewer metabolites which can affect the performance of downstream application if not completely removed during nucleic acid purification (Babu, 1997).

In the present study total RNA was isolated using trizol reagent. Trizol combines phenol and Guanidine thiocyanate in a monophase solution that facilitates the immediate inhibition of RNase activity. The homogenate was incubated at room temperature which allows nucleoprotein complexes to completely dissociate. Chloroform was added to remove chlorophyll and proteins. Even after chloroform extraction, there was formation of a flocculent white precipitate in the aqueous phase. This was likely due to precipitation of insoluble lipids and thus was remedied by adding additional chloroform and reextracting. Isopropanol was added to precipitate RNA which was then followed by ethanol (80%) wash. The pellet was dissolved in autoclaved DEPC treated water.

The modified procedure allowed the recovery of intact, high quality RNA from different treated leaves. Two distinct RNA bands corresponding to 28S, 18S were apparent in both the samples when analyzed on agarose gel (Plate10). In addition, the samples were quantified spectrohotometrically and their quality and quantity was assessed. The ratio for OD_{260}/OD_{280} for both samples was approximately 2.0 indicating pure RNA. The ratio for OD_{260}/OD_{230} was greater than 1.0 indicating pure RNA free from polysaccharides and polyphenols.

5.6 Preparation of cDNA

The total RNA from each treatment was treated with DNase I enzyme to eliminate traces of genomic DNA. Actual confirmation of complete degradation of genomic DNA in RNA preparation was done through PCR amplification using total RNA as template. There was no amplification from the total RNA preparation indicating absence of traces of genomic DNA as contamination.

However, elimination of contaminating genomic DNA enzymatically is very important in gene expression analysis using qRT-PCR (Chini *et al.*, 2007). Presence of genomic DNA/genetic copies of genes seriously alter the precision of expression quantitation of genes in target tissues.

5.7 Defence gene expression study

The expression of systemic Jasmonic Acid (JA) -related, systemic Ethylene (ET) -related responses and systemic Salicylic Acid (SA) -related defence genes in rice were determined in plant tissues of different treatment using semi quantitative RT-PCR and quantitative real-time RT-PCR. *Actine* gene was used as a house keeping control. In this investigation we focused on the three main hormone pathways known to be involved in plant defence, SA, JA and ET (Pozo *et al.* 2005; van Loon *et al.* 2006; Loake and Grant 2007), and their systemic response of the test pathogen upon pathogen *R. solani* infection to rice in presence of potent bioagent agent. It has been proved that many proteins are actively introduced into the plant and some of these effector proteins probably interfere with plant defence, not only in locally infected tissue, but also in systemic tissues through modulation of systemic signalling.

Much research has already been done to elucidate complex networks among different hormone-related defence pathways in Arabidopsis, and although some resemblances could be seen, observations from Arabidopsis cannot merely be translated into the rice–pathogen interactions.

Several defence proteins/genes have been identified in rice-microbes interaction through SAGE (Jantasuriyarat *et al.*, 2005).

In the current study, we have analysed the expression of genes involved in the systemic defence response (ISR and SAR) in rice-*R. solani*-bioagent (actinomycete) interaction model.

5.7.1 Differential activation ⁄ suppression of SA-, JA- and ET-regulated defence pathways upon pathogen infection and bioagent inoculation in rice

The JA biosynthesis gene *OsAOS2* showed differential expression in seed treatment of bioagent, combined treatment of bioagent and only bioagent treatment plants at 24hr, 48hr, 72hr and 120hr after pathogen inoculation, however it was down-regulated at 0hr after pathogen inoculation. Indeed, *OsJAMYB*, a specific JA-inducible transcription factor that is generally associated with fungal infection and host cell death (Lee *et al.* 2001), showed significantly higher expression at 120hr after pathogen inoculation in combined treatment of bioagent in comparison with healthy control plants which is upto 3.5 fold whereas expression of *OsAOS2* gene upto 1.5 fold at same condition. At 120hr after pathogen inoculation, expression of *OsJMT1* was highest upto 1.2 fold in combined treatment of bioagent.

OsAOS2, OsJMT1 and *OsJAMYB* showed the differential expression in seed treatment of bioagent, combined treatment of bioagent and only bioagent treatment plants at 24hr, 48hr, 72hr and 120hr after pathogen inoculation over the only treated control (pathogen) and healthy plant. Moreover, as already suggested above, this implied that the hormone regulated defence pathways are being up regulated by the actinomycete isolate IABT-A7 at this time point.

There is strong evidence demonstrating that AOS is a key enzyme and a control point of the JA biosynthetic pathway in *Arabidopsis*, a model dicotyledonous species. Contrary to our results the JA biosynthesis gene *OsAOS2* does not show differential expression in systemic tissues of migratory nematode-infected plants at 3 dpi, and is even down-regulated at 7 dpi (Kyndt *et al.*, 2011).

MeJA and its free acid jasmonic acid (JA), collectively referred to as jasmonates are important cellular regulators mediating diverse developmental processes, such as seed germination, flower and fruit development, leaf abscission, and senescence (Creelman and Mullet, 1995). An *Arabidopsis NTR1* homolog of *JMT* has been cloned to perform molecular biological studies in more detail. Characteristics of *JMT* indicated that the gene encodes an *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (JMT) a key enzyme for the jasmonate-regulated plant responses. Seo *et al.* (2001) proposed that a possible role of MeJA as an intracellular regulator and a long distance signal mediating intra- and interplant communications and also that JMT is a key enzyme for the jasmonate regulated plant responses. MeJA can act as an intracellular regulator, a diffusible intercellular signal transducer, or an airborne signal mediating intra- and interplant communications. Some signals generated during an early event of developmental processes or defense responses may activate *JMT* that can self amplify, stimulate or regulate its own expression, propagating the MeJA-mediated cellular responses throughout whole plants.

Similarly, ET biosynthesis and reception ⁄ response is slightly upregulated for example in case of *OsERF1* gene in seed treatment of bioagent, combined treatment of bioagent and only bioagent treatment plants at 48hr, 72hr and 120hr after pathogen inoculation over the only pathogen inoculated treatment and healthy plant but down-regulated at 24 hr after pathogen inoculation.

Unlike in case of *OsEIN2* highest up regulation was observed at 120hr after pathogen inoculation in seed treatment of bioagent, combined treatment of bioagent and only bioagent treatment plants over the only pathogen inoculated treatment and healthy plant.

A global analysis of Transcription Factor (TF) gene expression to identify candidate genes that may regulate disease resistance were studied (McGrath *et al.*, 2005). Their functional analysis of selected AP2/ERF genes has highlighted the value of this approach, demonstrating roles for both repressor- and activator-type ERFs in disease resistance and JA signaling. Additionally, several genes identified in their initial screen have also previously been implicated in plant defense (Berrocal-Lobo *et al.*, 2002; Brown *et al.*, 2003), while many other inducible genes do not yet have known functions.

In general, the hormone responses for SAR pathway observed upon pathogen inoculation such as *OsPR1b* was up regulated up to 12 fold in only pathogen treated sample at 48hr. First, the pathogenesis-related gene *OsPR1b*, which is generally seen as SAR marker gene is strongly up-regulated in seed treatment and combine treatment at 24hr. The gene *OsPR1b* was constitutively expressed at all the time interval in all the treatments over healthy control. Wherever bioagents are there the expression of PR1b treated control was not up regulated compared to treated control, so it indicates application of bioinoculants did not enhance SA pathway in the infected plant. Contrarily, bioinoculation induced/activated the genes which are the major players of ET and JA pathways.

OsNPR1 is important for BTH-induced and has been suggested to mediate the antagonistic cross-talk between the SA- and JA dependent pathways. Our data showed that *OsNPR1* is slightly up-regulated in seed treatment of bioagent, combined treatment of bioagent, only bioagent treatment and only pathogen inoculated treatment plants at 48hr, 72hr and 120hr.The presence of bioagent may allow to express the ISR gene throughout the time interval and also it prolong the expression of *NPR1* gene in bioagent treatment. Use of bioagent induced the expression of ISR gene in addition it prolong the expression of NPR1 the intermediate key factor of defence pathway.

Thus, the study has indicated that actinomycete isolate IABT-A7 has multiple beneficial traits. The actinomycete isolate IABT-A7 performed well against different isolates of *R. solani in vitro* and *in vivo* and also in enhancing the plant growth, reducing the disease incidence, inducing systemic resistance as evidenced by enhanced production of defense genes (ISR activity). The seed, soil and foliar application of actinomycete isolate IABT-A7 was controlled disease sheath blight of rice as well as enhanced plant growth. However, it showed that the differential expression of defence genes specifically related to ISR pathway in actinomycete isolate IABT-A7 treated samples at different time intervals after pathogen inoculation.

The key genes involved for ISR pathway *viz.*, *OsAOS2, OsJMT1* and *OsJAMYB* were up regulated in this *R. solani*, rice and actinomycete isolate IABT-A7 interaction. The master regulator gene of defence pathway *OsNPR1* was constantly expressed in all treatment at all time intervals. Our result strengthens the ISR inducing ability of IABT-A7 isolates and biocontrol potential of the isolate at molecular level.

SUMMARY AND CONCLUSIONS

The present investigation an attempt was made to identify of potential biocontrol isolates of *Trichoderma*, *Rhizobacteria* and Actinomycets against rice sheath blight pathogen, *Rhizoctonia solani* and to study of expression of defence genes/ PR genes in host in presence of bioagent and the pathogen. The specific objectives were to isolate and identify native actinomycete strains from rice rhizosphere. *In vitro* screening of *Trichoderma*, *Rhizobacteria* and actinomycete against *R. solani* isolates was done to elucidate their mechanisms of biocontrol. The ability of the strains to reduce disease incidence and to promote plant growth was also assessed in glasshouse conditions. It also envisaged the molecular interaction among rice- *R. solani*-actinomycete (IABT-A7) by studying expression pattern of defence genes. The results obtained in the present investigation are summarized below.

1. Actinomycete isolates were enumerated from rhizosphere soil of healthy rice plants from Mugad.
2. A total eleven colonies were putatively selected by observing morphological characters which were purified and preserved in - 80^0C.
3. Rapid method of identification of actinomycete to the genus level was done for all eleven isolates by ARDRA. ARDRA profile revealed three isolates as *Actinopolymorpha* spp. (IABT-A1, IABT-A6 and IABT-A7) and remaining eight isolates belongs to *Streptomyces* genera.
4. In this study, a quick method has been developed for highly consistent and reproducible inoculation of sheath blight pathogen to rice and for more accurate measurement of disease severity.
5. A total of fifty bioagents *viz.Trichoderma*, pseudomonads, PPFM and actinomycete isolates were selected for *in vitro* screening.
6. Among different *Trichoderma* isolates, the highest per cent inhibition was observed in *T. virens* (1002) as (73.11%), *T. virens* (1010) as (73.10%) against *R. solani* isolates. Similarly, for pseudomonad strain (19) showed 100 per cent inhibition and 12, 20, 6, 25, 22, soy6 showed 90-100 per cent inhibition against *R. solani* isolates. In case of *Methylobacterial* strains, 38U only showed highest inhibition as 83.33 per cent against *R. solani* isolates. Further, the actinomycete stains IABT-A2, IABT-A3, IABT-A7, and IABT-A8 showed total 100 per cent inhibition against *R. solani* isolates.
7. The efficient bioagents which performed well *in vitro* were taken for *in vivo* assay *viz.*, *T. virens* (1002), *T. virens* (1010), *T. harzianum* (1034), *T. harzianum* (1036), *T. harzianum* (1040) from *Trichoderma* isolates, 12,19,20,soy2 and soy6 from pseudomonad strains and IABT-A1, IABT-A2, IABT-A6, IABT-A7 and IABT-A8 from actinomycete isolates.
8. Of the fifteen efficient bioagent isolates tested, combined treatment of IABT-A7 reduced the disease parameter *viz.*, lesion length and number of dried leaves and enhanced the plant growth parameters *viz.*, the plant height, number of tillers, root length, root biomass in glasshouse condition. IABT-A7 was taken for further molecular studies.
9. Induced systemic resistance (ISR) was assessed through expression profiling of defence genes in rice by performing real-time PCR. The total RNA was isolated from the treatment as seed treatment of IABT-A7, combined treatment of IABT-A7,only bioagent (IABT-A7) treatment, treated (pathogen) control and untreated (healthy) control at different time interval as 24 h, 48 h,72 h and 120 h after infection with pathogen.
10. The total RNA was treated with DNase I for elimination of genomic DNA contamination. Actual confirmation of complete degradation of genomic DNA in RNA preparations was done through agarose gel electrophoreses containing formaldehyde analysis, where in two bands were observed *viz. 28S rRNA* and *18S rRNA*.
11. The variance estimation, M value, which should be < 1.5 for a stably expressed gene, was in the order of magnitude 0.301 and 0.306 for *actin* (*AC1*) and *EF-la* respectively. Based on M value *actin* (*AC1)* gene was selected as a reference gene for experiment.
12. The expression of systemic Jasmonic Acid (JA) -related defence genes *viz.*, *OsAOS2, OsJMT1* and *OsJAMYB* showed the differential expression in all the treatments and at all time interval over the respective treated and untreated control.

13. ET biosynthesis related defence genes *viz.*, *OsERF1* is slightly upregulated in all treatments at 48 h, 72 h and 120 h after pathogen inoculation over treated control and untreated control but down-regulated at 24 h after pathogen inoculation. Unlike, *OsEIN2* highest up regulation was observed only at 120 h in all treatment over treated and untreated control.

14. The hormone responses for SAR pathway observed upon pathogen inoculation such as *OsPR1b* was up regulated up to 12 fold in only pathogen treated sample at 48 h.

15. The master regulator gene of defence pathway *OsNPR1* was constituently expressed in all treatment at all time intervals over untreated control.

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APPENDIX

1. Starch Casein Agar media (SCA) (Anisha *et al*., 2006)

Sl. No.	Name of components	Quantity (g)
1	Soluble starch	10
2	Casein, Vitamin free	0.3
3	Potassium nitrate	2
4	Sodium chloride	2
5	di-potasssium hydrogen phosphate	2
6	Magnesium sulphate, hepta hydrate	0.05
7	Calcium carbonate	0.02
8	Ferrous sulphate, hepta hydrate	0.01
9	Agar	10
10	Distilled water	1000 ml

pH- 7.2

2. X Starch casein agar media

Sl. No.	Name of components	Quantity (g)
1	Soluble starch	1
2	Casein, Vitamin free	0.03
3	Potassium nitrate	0.02
4	Sodium chloride	0.02
5	di-potassium hydrogen phosphate	0.02
6	Magnesium sulphate, hepta hydrate	0.005
7	Calcium carbonate	0.002
8	Ferrous sulphate, hepta hydrate	0.001
9	Agar	18
10	Distilled water	1000ml

pH- 7.2

3. Soybean-Casein Digest Agar Medium (SCBA) (Basavaraj *et al*., 2010)

Sl. No.	Name of components	Quantity (g)
1	Pancreatic digest of casein	15
2	Soya peptone	5
3	Sodium chloride	5
4	Agar	15
5	Distilled water	1000 ml

pH- 7.3

4. Nutrient Broth (Vijaykumar *et al*., 2010)

Sl. No.	Name of components	Quantity (g)
1	Beef extract powder	2
2	Yeast extract powder Type I	2
3	Peptone	5
4	Sodium chloride	8
5	Distilled water	1000 ml

5. Luria broth (Maniatis *et al.*, 1982)

Sl. No.	Name of components	Quantity (g)
1	Tryptone	10
2	Yeast extract powder Type I	5
3	Sodium chloride	5
4	Distilled water	1000 ml

6. Extraction buffer for actinomycetes DNA isolation (Sambrook, 2001)

Name of component	Concentration
Tris-Cl (pH 8.0)	100mM
Sodium chloride	250mM
EDTA (pH8.0)	200mM
Water	Make up the volume

Buffers and solutions for Agarose Gel Electrophoresis (Sambrook, 2001)

1. Recipe for 0.7 per cent agarose gel (40 ml)

Name of the component	Quantity
Agarose	280 mg
1X TAE	40 ml
EtBr (10mg/ml)	0.5µg

2. 50X TAE Buffer

Name of the component	Quantity
Agarose	280 mg
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml
Water	Make up the volume to 1000 ml

3. 6X Loading Dye

Name of the component	Quantity
Bromophenol Blue	0.25 %
Sucrose	40.0 %
Water	Make up the volume

ANTIPHYTOPATHOGENICITY OF BIOAGENTS AND EXPRESSION ANLYSIS OF SELECTED DEFENCE GENES IN RICE IN PRESENCE OF ACTINOBACTERIA AND *Rhizoctonia solani*

2013

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ABSTRACT

A total of fifty bioagent isolates comprising *Trichoderma*, pseudomonad, PPFM and actinomycete spp., were screened against rice sheath blight pathogen *Rhizoctonia solani* under *in vitro* condition and potent isolates were selected for in vivo assay. Five potent isolates from each of the biocontrol agent was further evaluated against *R.solani* under glasshouse condition. Of the fifteen efficient bioagent isolates evaluated, combined treatment of Actinomycete isolate (IABT-A7) was promising with reduced disease parameters, lesion length and number of dried leaves and enhanced the plant growth parameters *viz.*, the plant height, number of tillers, root length, root biomass. Potential actinomycete (IABT-A7) isolate was identified as *Actinopolymorpha* spp., through Amplified rDNA Restriction Analysis (ARDRA).

Expression of the key genes involved in Induced Systemic Resistance (ISR) and Systemic Acquired Resistance (SAR) were analysed by real-time PCR. Gene expression and quantum of expression was profiled at different time intervals 24 hr, 48 hr,72 hr and 120 hr after infection of pathogen from treatments *viz.*, seed treatment of IABT-A7, combined treatment of IABT-A7 (seed+soil+foliar),only bioagent (IABT-A7) treatment, treated control (only pathogen) and healthy plant. Jasmonic Acid (JA) pathway related genes, *OsAOS2*, *OsJMT1, OsJAMYB* showed the differential expression in all the treatments and at all time intervals over the treated control. ET biosynthesis gene, *OsERF1* was up regulated in all treatments at 48 hr, 72 hr and 120 hr over treated control. The relative up regulation of these genes was highest in combined treatment over control. *OsPR1b,* a key gene for SAR pathway was up regulated in only pathogen treated samples and up regulated up to 12 fold at 48 hr. The master regulator defence gene *OsNPR1* was constantly expressed at all intervals over untreated plant.