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Characterization and Utilization of Rice Defense Associated with Partial Resistance to Bacterial Panicle Blight: An Emerging Rice Disease Problem in the Southeastern United States

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CHARACTERIZATION AND UTILIZATION OF RICE DEFENSE SYSTEM ASSOCIATED WITH PARTIAL RESISTANCE TO BACTERIAL PANICLE BLIGHT: AN EMERGING RICE DISEASE PROBLEM IN THE SOUTHEASTERN UNITED STATES

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DEDICATION

To my parents Ratna Lal Shrestha, Shanta Laxmi Shrestha and Prem Kumari Shrestha….

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ABSTRACT

Rice is a staple food for much of the world's growing population. Rice production is limited by a number of abiotic and biotic factors. These factors have direct effects on food security because less food is available to growing population.

Bacterial panicle blight (BPB), caused by the bacterium *Burkholderia glumae,* is an emerging disease that causes grain rot, panicle discoloration, and unfilled grains in rice. Up to 70% of yield reduction has been reported in severely infected rice fields. No completely resistant rice cultivars have been identified, however, a medium-grain cultivar, Jupiter, showed a high level of partial resistance to this disease. A research was conducted to characterize and utilize the rice defense system associated with partial resistance to BPB. Various chemical compounds and biological agents were used to enhance the rice defense system and *in vitro* control of *B. glumae,* respectively.

Microarray studies, done by Dr. Nandakumar and Dr. Rush, showed that several defense related genes and transcriptional regulators were highly up-regulated in Jupiter and slightly up-regulated in Trenasse, a susceptible long-grained cultivar, when challenged with *B. glumae*. Induction of the expression of those genes in Jupiter and Trenasse were verified by reverse-transcription PCR. Genes encoding an NAC-like transcription factor (NTF) and a grain filling protein, prolamin, was highly induced in Jupiter but not in Trenasse under different treatments of *B. glumae* and its mutant derivatives. These genes may be involved in the partial resistance to BPB, and could be used as a genetic marker and breeding tools to develop BPB resistant rice cultivars.

In an attempt to develop control measures for BPB in rice, several chemicals, including jasmonic acid, salicylic acid, ascorbic acid, 2, 6-dichloroisonicotinic acid, and ethylene, known

to induce plant defense systems against various plant pathogens were tested for their ability to enhance rice defense systems and reduce BPB development. Results showed that pretreatment of rice with ascorbic acid significantly suppressed BPB development while only minimally reducing yield. In the meantime, several biological agents isolated from rice leaves showed antagonistic effect on *B. glumae*, and *Rhizoctonia solani*, the causal agent of sheath blight in rice.

1. INTRODUCTION

Rice (*Oryza sativa* L.) is a major food cereal for majority of world population. It provides a larger portion of total calories for half of the world's population (www.usarice.com). There are two subspecies of rice, indica and japonica, cultivated worldwide. Indica rice is dominated over japonica because the earlier one cooks dry and separately, while the later cooks sticky and moist. Japonica rice is more rounded compared to the indica rice cultivar (Childs and Burden, 2000). Rice can be successfully grown in broad range of environments. In the U. S. rice is grown in six states, Arkansas, Mississippi, Louisiana, Texas, California and Missouri (Federation, 2011)(USA Rice federation, http://riceinfo.com/all-about-rice/types-of-rice). The U. S. is one of the major exporters of rice in the international markets. Wide-spread development of major rice diseases results in the reduction of the rice (4.36 tons/ha in 2008 to 4.32 tons/ha in 2009), although the total harvested area increased by 560,632 hectares (http://faostat.fao.org/site/567/default.aspx#ancor). Due to such yield reductions, the availability of rice to ever growing population will be less. So, it is necessary to increase rice production from the limited area of the land to maintain the supply. The production of rice is affected by various abiotic factors and biotic factors. The abiotic factors include soil fertility, agricultural inputs, crop management, and the growing environment, whereas biotic factors include various insects, viral, fungal, and bacterial diseases.

Bacterial panicle blight (BPB) is an emerging rice disease in the rice producing area throughout the United States except in California. Brown or straw-colored discoloration of rice panicles, but not the panicle branches; spikelet sterility due to the florets abortion which results in unfilled grains with erect panicles instead of bending over (Appendix A); and reduction of tillers which results in yield reduction are the characteristic symptoms developed by BPB. Previously, it was thought to be caused by abiotic factors like high night temperature, toxic

chemicals and water stress. However, later it has been reported that *Burkholderia glumae* (previously *Pseudomonas glumae*) is the major causal organism of BPB in Louisiana and its neighboring rice producing southern states (Shahjhan et al., 2000). About 60% of Louisiana rice fields were affected by BPB (Gonzalez et al., 2007; Shahjhan et al., 2000). Infected seeds from the previous year provide the source of inoculum. Colonization of the leaf sheaths by the pathogen provides the primary source of inoculum (Tsushima et al., 1991). However, the frequency and the severity of infection of the flag leaf sheath provides the estimation of the disease infection on the panicles (Tsushima et al., 1996).

High temperature and high humidity at the flowering stages provide favorable environment for epidemics of BPB (Tsushima et al., 1995). It has been reported that there were severe outbreaks of this disease in the southern rice production states of the U. S. in the years 1995, 1998, and 2000 that resulted in about 40% yield reduction in most of the infected fields (Nandakumar et al., 2009; Shahjhan et al., 2000). High temperatures and high humidity were recorded during these years, along with high night temperatures. So, higher temperatures during the growing seasons will facilitate the occurrence of the diseases. It has also spreading into other rice growing regions of the world including the Philippines, Korea, Vietnam, and Taiwan (Cottyn et al., 1996; Jeong et al., 2003; King et al., 1954 ; Trung et al., 1993). The optimal temperature for the growth of the pathogen ranges from 30-35°C (Kurita et al., 1964). Due to the current global warming, the incidence of the BPB may be severe in tropical regions (Schaad, 2008).

The major causal agent of BPB, *B. glumae*, was first reported in Japan causing rice grain rot and seedling rot (Goto and Ohata, 1956). Previously this pathogen was classified in the genus *Pseudomonas*, but later seven species of this genus (*P. solanacearum, P. caryophylli, P. cepacia*,

P. gladioli, P. mallei, *P. pickettii*, *P. pseudomallei*) were reclassified as *Burkholderia* (Yabuuchi et al., 1992) on the basis of rRNA homology group II as a new genus. In 1994, two plant pathogenic bacteria, *P. glumae* and *P. plantarii* were transferred to the genus *Burkholderia* (Urakami et al., 1994). About 66 described species were listed in the genus *Burkholderia* to date (http://www.bacterio.cict.fr/b/burkholderia.html). Among those species, *B. glumae* is one of the pathogens which cause seedling rot, sheath rot, and grain rot (Goto and Ohata, 1956; Goto et al., 1987). *B. gladioli* is another species from the genus *Burkholderia,* frequently found in infected rice panicles, but it is less virulent than *B. glumae* (Nandakumar et al., 2009).

B. glumae is a gram negative, rod-shaped, aerobic, non-sporing bacterium. It has one to four polar flagella, which helps in motility of bacteria. The colony of this bacterium is characterized by yellow color. Yellow pigment is due to the water-soluble pigment produced by the bacteria and is media dependent (Urakami et al., 1994). It produces the phytotoxin, toxoflavin, which is a major virulence factor; and is regulated by the quorum-sensing (Kim et al., 2004). *B. glumae* has a wide host range and causes wilting in many field crops such as tomato, sesame, eggplant, and perilla (Jeong et al., 2003). It is a seed-borne pathogen and is detected in the epidermis and parenchyma of the infected seeds (Hikichi et al., 1993). Primarily, the pathogen enters through the stomatal openings to the lemma and paleae of the rice seed, and multiplies in the intercellular space of the cells (Tabei et al., 1989). For the long distance movement, this pathogen uses the vascular system of the plant (Yuan, 2004).

Use of pathogen-free seed is the major control measure of this disease (Saichuk, 2009). However, there are some chemical and biological control practices methods for this disease. Oxolinic acid, a quinoline derivative, is used to treat seed for disease control. It inhibits the supercoiling activity of the DNA gyrase, and DNA synthesis in bacteria is inhibited (Drlica and

Zhao, 1997). However, the use of oxolinic acid is not common in most parts of the world. Moreover, *B. glumae* itself develops resistance against this antibiotic (Maeda et al., 2004). Additionally, various biological agents are also found to be effective in controlling this disease including *Bacillus* species, and avirulent strains of *B. glumae* (Furuya et al., 1991). Host plant resistance is the most important control measures for bacterial panicle blight. A completely resistant rice cultivar for this disease has not yet been identified. However, some of the cultivars including Jupiter, a medium-grained cultivar (Sha et al., 2006), and LM-1, a mutant rice line (Groth et al., 2007), show relatively high levels of partial resistance to BPB (Shahjhan et al., 2000).

Plants are continuously facing different types of abiotic (drought, salinity, high and low temperature, etc.) and biotic (insect feeding, pathogen attack, etc.) stresses. To confront those challenges, plants develop efficient mechanisms including basal defense and induced defense system. Plants respond to them with proper physiological, biochemical, and morphological changes. Basal defense system occurs during the early stage of plant-pathogen interactions while induced defense system is activated after pathogen attack (Agrios, 2005). Interaction between an avirulence (*avr*) gene of pathogen and a corresponding resistance (*R*) gene of the host produces resistance to the disease as a result of incompatible reaction, also known as "gene-for-gene resistance" (Dangl and Jones, 2001; Flor, 1955). Each *R* gene specifically recognizes only one specific elicitor produced by the pathogens, and those recognitions will trigger an effective defense reaction which leads to the prevention of growth and development of pathogen in the host cells. *R* gene-mediated resistance shows effective defense responses including hypersensitive response (HR), which is localized cell death of host preventing from the further

spread of pathogen. HR triggers the activation of salicylic acid-dependent signaling, which will increase the SA accumulation and ultimately activate defense genes (Glazebrook, 2005).

In the absence of specific resistance, non-specific resistance known as basal resistance will be initiated (Pozo et al., 2004). The induction of such basal plant defense responses is mediated by signaling pathways, and results in acquiring resistance throughout the whole plant system gradually. This is called as systemic acquired resistance (SAR). SAR protects the plant from a broad range of pathogens, including bacteria, fungi, virus, nematodes, and reduces the diseases. It is associated with the activation of various defense-related genes, pathogenesis-related (PR) proteins, and several families of transcription factors.

Different types of defense related genes, pathogenesis-related (PR) proteins and transcriptional regulators are up-regulated or down-regulated in response to pathogens. A form of defense reaction is the rapid formation of reactive oxygen species (ROS) like superoxide, hydrogen-peroxide, hydroxyl radical, and oxygen. These ROS are involved in signal transduction and limiting pathogen access in plants (Barna et al., 2003). ROS are produced by partial reduction of molecular oxygen in higher plants. Antioxidants, like ascorbate, proline and glutathione, are important redox signaling components and provide crucial information on cellular redox state that control gene expression linked with biotic and abiotic stresses (Shao et al., 2008). Several antioxidants are up-regulated by hypersensitivity reactions (Barna et al., 2003). In addition to those antioxidants, different types of antimicrobial and grain filling proteins, transcription regulators are positively and/or negatively regulated in response to biotic and abiotic stresses. Small, cystein rich, basic polypeptides are also involved in the defense mechanism of both plants and animals (Epple et al., 1997).

In addition to pathogens, various natural and synthetic chemical compounds can also elicit similar plant defense responses. Salicylic acid (SA), jasmonic acid (JA), ethylene (ET), mediated expression of defense mechanisms and disease resistance has been extensively studied in the dicotyledonous plant *Arabidopsis* and tobacco (Glazebrook, 2005; Yang et al., 1999). However, molecular and genetic studies in the monocotyledons have not been studied as much as compared to dicotyledonous plants. Rice is used as a monocot model system among the cereals to study the disease resistance mechanisms and pathways. Disease resistance pathways in rice are different in many ways from those in dicots. For instance, the basal levels of SA in rice are higher than dicots, and no change will occur in the SA after the infection with virulent or avirulent pathogens (Chern et al., 2005a; Silverman et al., 1995). Induction of several *R* genes has been reported to be expressed in response to several pathogens in rice, which is one of the main defense mechanisms in plants. It encodes the protein containing central nucleotide binding region (NB) and a C-terminal leucine rich repeat (LRR) (Dangl and Jones, 2001) that form a sub group with in the signal transduction ATPases with numerous domains (STAND) family (Lukasik and Takken, 2009), which bind and hydrolyze nucleotides. Coiled-coil domain (CC-NB-LRR) is one of the NB-LRR proteins, identified in rice (Pan et al., 2000). *NLS1*, a CC-NB-LRR type *R* gene, is involved in the activation of defense response in rice against bacterial pathogen *Xanthomonas oryzae* p.v. *oryzae*, including cell death (Tang et al., 2011).

Incompatible host-pathogen interactions cause HR due to local cell death around the infection region which inhibits the spreading of pathogens. In some of the cases, those localized cell death could lead to the activation of defense response in the whole plant system. It is involved in the induction of expression of *PR* proteins that makes plants resistant to broad range of bacterial, fungal as well as viral pathogens (Ryals et al., 1996). This type of resistance is

systemic acquired resistance (SAR). Smith and Meatraux (1991) reported that infection of rice with *Pseudomonas syringae* pv. *syringae* induces SAR to *Pyricularia oryzae*. Induction of *NH1,* a homolog of *Arabidopsis NPR1,* in rice confers resistance to *X. oryzae* pv. o*ryzae* (Chern et al., 2005b). *Magnaporthe oryzae* and *Cochliobolus miyabeanus* infection in rice induces HR in resistant cultivars of rice, (Ahn et al., 2005). Several defense pathways mediated by defense related genes (Lee et al., 2001) and pathogenesis related (*PR*) genes including *PR1a*, *PR1b* (Agrawal et al., 2001), and *PR5* (Mei et al., 2006; Rakwal and Komatsu, 2000) are induced in rice in response to various bacterial and fungal pathogens, and exogenous application of various signaling molecule. Exogenous application of JA activates defense gene expression in rice seedlings against the rice blast fungus *M. grisea,* increasing the production of phytoalexins and other chemical compounds. Furthermore, overexpression of rice the allene oxide synthase gene induces expression of *PR* genes such *PR1a*, *PR3*, and *PR5*. This overexpression also increases JA level endogenously and makes the plant resistance to *M. grisea* (Mei et al., 2006). However, it has also been reported that the endogenous levels of the JA did not increase significantly. In contrast, exogenous application of JA only induces the resistance against *M. grisea* in the systemic leaves (Schweizer et al., 1998).

Several molecules including transcription factors and protein kinase are involved in signaling pathways (Fujita et al., 2006). *OsNAC6,* one of the orthologue of Arabidopsis *ATAF2*, is highly induced by wounding, exogenous application of methyl jasmonate and SA, but not by abscisic acid (Delessert et al., 2005). *OsNAC6* may act as an activator of PR proteins in rice because overexpression of *OsNAC6* gene in rice up-regulates various biotic-stress related genes including PR proteins(Nakashima et al., 2007).

As previously mentioned, transcription factors are involved directly or indirectly in regulating plant defense responses against pathogens. Those transcription factors were categorized into different families such as: NAC, WRKY, ERF, MYB, on the basis of conserved structural domains that are involved in DNA binding activity. These transcription factor families are involved in regulation of defense responses in plants (Delessert et al., 2005; Eulgem and Somssich, 2007; Wang et al., 2009). NAC consists of a large family of plant specific transcription factors. NAC protein of this family is specific to plants, and includes a conserved N-terminal DNA binding domain and a variable C-terminal domain (Xie et al., 2000). *A. thaliana* have more than hundreds of NAC coding genes. NAC is derived from three different genes NAM (no apical meristem), ATAF (Arabidopsis transcription activation factor) and CUC (cup-shaped cotyledon), which contain a conserved NAC domain. Rice NAC family can be categorized into five groups. Group I can be further classified into five sub-groups which are related to development process, group II is more complex than group I, and is also classified into few subgroups. Group III, on the other hand, is related to stress related NAC, while group IV and V have fourteen and two NAC members of rice, respectively (Fang et al., 2008).

Overexpression of *SNAC1*, *SNAC2*, and *OsNAC6* enhances drought tolerance and also blast resistance in rice (Hu et al., 2006; Nakashima et al., 2007; Zheng et al., 2009). Involvement of NAC proteins in biotic and abiotic stress responses is implied by the induction of potato *StNAC* gene by *Phytophthora infestans* infection and *Brassica napus* NAC genes by fungal and insect-pests infection. Overexpression of *A. thaliana* NAC genes increased the drought tolerance (Collinge and Boller, 2001; Olsen et al., 2005; Ren et al., 2000). Overexpression of stress responsive NAC gene, *SNAC1*, in rice increased the stomata closure and drought resistance in drought environmental condition (Hu et al., 2006). Similarly, *HvNAC6* in barley involves in

penetration resistance in barley against *Blumeria graminis* f. sp. *hordei* (Jensen et al., 2007). NAC transcription factor, such as *ONAC045*, is induced by abiotic stress and act as transcriptional activator (Zheng et al., 2009). Furthermore, another NAC transcriptional factor, *OsNAC4*, mediates the induction of HR cell death along with the typical morphological changes by plant differentiation in rice when challenged by the pathogen (Kaneda et al., 2009). *ATAF1* is negatively involved in showing disease resistance. Overexpression of *ATAF1* in *Arabidopsis* shows increased susceptibility to *Botrytis cinerea, P. syringae* pv. *tomato* DC3000 and *Alternaria brassicicola*. Several defense related genes are also down regulated in *ATAF1* overexpressed *Arabidopsis* (Wang et al., 2009). Furthermore NAC proteins play a role in resistance against viral disease in rice and other economically important crops. Disruption of NAC protein in rice shows enhanced resistance against rice dwarf virus (Yoshii et al., 2009).

WRKY super family of transcription factor is also involved in controlling the transcription of various JA and SA responsive defense-genes by expressing after the infection with various bacterial and fungal diseases of rice (Kim et al., 2000; Ryu et al., 2006; Wen et al., 2003). OsWRKY1-associated defense resistance directly or indirectly regulates the expression of several genes involved in various physiological processes, and also have crosstalk with the SNAC1-mediated abiotic stress defense pathway (Qiu et al., 2008). It also regulates the defense-related genes such as *PR1a* (acidic PR protein) and *LOX* (lipoxygenase) and *PR10* (Qiu et al., 2007; Shimono et al., 2007). *OsPR1a* and *OsPR1b* genes are expressed as defense responses when challenged by the blast pathogen *M. grisea* in rice (Agrawal et al., 2001).

About 80 percent of total seed protein is occupied by glutelins, so they are the major storage proteins in rice seeds. Rice glutelins belong to the globulin family, however, they are insoluble in salt solution (Krishnan et al., 1992). In addition to glutelins, rice seeds comprise

alcohol-soluble proteins and prolamins in the endosperm and contain high percentage of glutamine residues; furthermore, sulphur-rich globulins are also accumulated in rice endosperm during the development of rice seeds (Krishnan et al., 1992). Previous microarray data suggested that these seed storage proteins in Jupiter cultivar were induced after the infection of *B. glumae*, however, they were not induced in the susceptible cultivar Trennase as compared to non-infected rice plants (Nandakumar and Rush, 2008).

Additionally, several natural and synthetic chemical compounds can induce similar plant defense responses as plant pathogens via SAR (Kessmann et al., 1994). The movement of SAR from the infected regions of the plants to the uninfected regions occurs through the phloem (Dempsey et al., 1999). Methyl salicylate (MeSA), derivative of SA helps in the mobility of defense signal for SAR, however, SA also induces cell death in the presence of reactive oxygen species (ROS) and nitric oxide (NO) (Vlot et al., 2009). SA widely known functions in plant resistance responses and plays a role in the induction of hypersensitivity response (HR). SA is involved in both local defense reactions at infection sites and the induction of systemic resistance (Durner et al., 1997)

In addition to SA, JA - mediated and ET - mediated signaling pathways are also involved in the regulation of defense mechanism (Smith et al., 2009). These two molecules in addition to another molecule, SA, interact with each other to induce the expression of various genes responsible for defense system. However, JA is also known as an essential signaling molecule for developmental processes, e.g. pollen maturation, flower and fruit development, photosynthesis, senescence and root growth (Turner et al., 2002), and defense and stress responses of monocots (Agrawal et al., 2001). JA plays a vital role in the production of secondary metabolites in plants at cellular level and eliciting the phytoalexin production in rice

leaves as well (Rakwal et al., 1996). JA dependent responses are related with enhanced expression of several defense genes, which encode PR proteins, thionin and plant defensins (Pieterse and van Loon, 1999). Rakwal and Komatsu, (2000) reported that rice plants respond to the inoculated blast fungus (*Magnaporthe grisea*) by inducing the expression of *OsPR1* transcripts. Exogenous application of JA induced the accumulation of mRNA of *PR1, PR2,* and *PR3* in rice. However, JA did not accumulate upon the infection with either compatible fungal pathogen *M. grisea* or the incompatible bacterial pathogen *P. syringae* pv *syringae* (Schweizer et al., 1997). The effect of JA on plant-pathogen interactions in potato and tomato, by exogenous application showed the induction of SAR against *Phytophthora infestans* (Cohen et al., 1993).

These signaling molecules are specific in nature, and their induction pattern at transcript levels may be variable. For example, expression patterns of individual genes in response to SA, JA and ET in monocots like rice are different from those in dicot plants. Furthermore, transcript levels of each gene differed in leaves, roots, and flowers accordingly to each gene (Mitsuhara et al., 2008). The expression of lipoxygenase, which catalyzes the biosynthesis of JA from α-linolenic acid, is the defense gene product in the leaves of diseased-rice which is correlated with resistance to *M. grisea* (Ohta et al., 1991). SA and hydrogen peroxide strongly induced mRNA level of *OsPR1* genes, while ABA induced moderately (Agrawal et al., 2001).

Complete resistance is characterized by the prevention of the pathogen reproduction in incompatible combinations of host and pathogens which is controlled by a single gene. However, partial resistance reduces the level of reproduction of pathogens even in compatible interaction (Parlevliet 1988). Most of the partial resistance is stable in environment with different pathogens for long period of time because it is non-race specific, and quantitative and polygenic. In contrast complete resistance is governed by single major gene and can be overcome within a few years

due to the evolution of new races of same pathogens (Zenbayashi et al., 2002). Due to the unavailability of suitable chemical and biological control for BPB, host-plant resistance will serve as a source of breeding tools for the development of BPB resistant cultivar in the future.(Zenbayashi et al., 2002)

In this study, rice defense system against *B. glumae* and the alternative method of controlling BPB symptoms in rice were examined. To study the defense responses of rice to the *B. glumae* and its mutant derivatives, deficient in toxoflavin production, and deficient in toxoflavin production and functional type III secretion system strains, expression analysis was done by using RT-PCR on various defense-related genes, transcriptional activation and two grain-filling protein encoding genes identified previously by microarray analysis (Nandakumar and Rush, 2008), using rice cultivars that show partial resistance or susceptibility. Exogenous application of several signaling molecules and *in vitro* assays of several endophytes isolated from heading stage of different rice cultivars were also studied.

2. A MEDIUM-GRAINED CULTIVAR, JUPITER, SHOWS A HIGH LEVEL OF PARTIAL RESISTANCE TO BACTERIAL PANICLE BLIGHT (BPB)

2.1 MATERIALS AND METHODS

2.1.1 Plant Materials

The rice cultivars used in this experiment were, a medium-grained partial resistant cultivar, Jupiter (Sha et al., 2006), and a long-grained susceptible cultivar Trenasse. Those cultivars were grown on a mixture of sterilized soil and sand in a proportion of 2:1 in a pot, in greenhouse of the Louisiana State University campus in late spring 2009. Each cultivar was grown in three pots and planted repeatedly for three times.

2.1.2 Bacterial Inoculation

B. glumae 336gr-1, a virulent and reference wild type strain, and two of its derivatives: a) toxoflavin deficient mutant *B. glumae tox*$^-$ and b) both toxoflavin and HR deficient mutant *B. glumae tox*$^-$/*hrp*$^-$ (Table 2.1) were used in this study. Pure cultures of strains were streaked heavily on King's B media (20 g of proteose peptone (Difco), 1.5 g K_2HPO_4, 1.5 g $MgSO_4.7H_2O$, 10 ml glycerol, 15 g agar in one liter of distilled water) and incubated at 37°C one day prior to inoculation. About 50 ml of bacterial suspensions of $0.1OD_{600}$ (1×10^8 CFU/ml) were prepared in sterile ddH_2O. After plants reached about 30% of heading stage, *B. glumae* strains were inoculated on panicles till it begins to drip down in each pot of both cultivars. Inoculation was done with a hand sprayer separately for each pot of plants and kept separated until the inoculum dried or about one hour, to inhibit contamination of bacteria between the treatments or healthy plants. Along with bacterial strains, sterile ddH_2O was also sprayed as a negative control. There were four treatments and three replications with two different cultivars of rice.

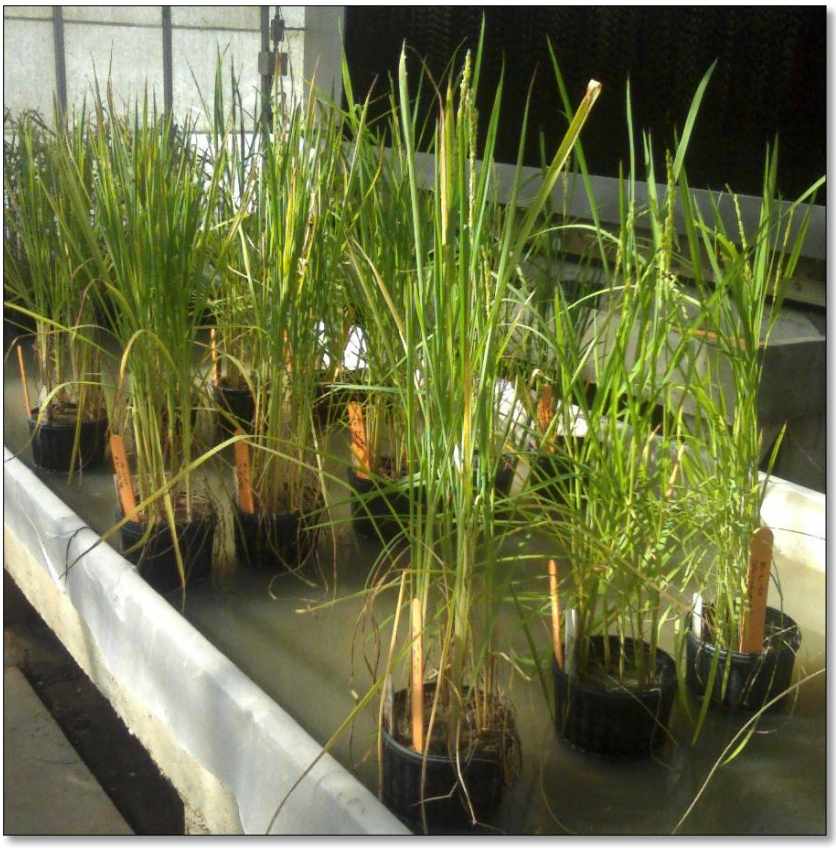

Figure 2.1 Arrangement of pots with four different treatments; sterilized ddH_2O as a negative control, *B. glumae* 336gr-1, *B. glumae tox*$^-$ and *B. glumae tox*$^-$/*hrp*$^-$, and three replications in the greenhouse.

2.1.3 Disease Severity Assessment and Panicle Sample Collection

Diseases symptoms were scored daily for 10 days, in each treatment. Disease severity was scored by using a standard scale of 0 to 9; 0 = no disease symptoms, 1 = 1 to 10% disease symptoms in the panicles, 2 = 11 to 20% disease symptoms, 3 = 21 to 30% disease symptoms, 4 = 31 to 40% disease symptoms, 5 = 41 to 50% disease symptoms, 6 = 51 to 60% disease symptoms, 7 = 61 to 70% disease symptoms, 8 = 71 to 80% disease symptoms, 9 = more than 80% disease symptoms. Diseases severity was calculated as ∑ (number of samples with each rating × rating value)/total number of panicles (Devescovi et al., 2007). Additionally, samples

from the panicles of each of the treatment were excised at day 0, day 2, day 3, and day 4, packed in labeled aluminum foil, and brought into the laboratory in a container with liquid nitrogen, and stored at -70 °C for further processing.

2.1.4 Total RNA Extraction

Total RNA of the preserved panicle samples stored at -70°C was extracted using RNAeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. The extracted total RNA was quantified with Nano Drop ND-1000 Spectrophotometer (Nano Drop, Wilmington, DE). The total RNA was diluted to 100 ng/µl, and stored at -70 °C.

2.1.5 Primer Design

Primers for defense-related gene, defensin, NAC-like transcription factor (NTF) encoding gene (Os01g0393100); NAC4 (Os01g0816100) grain-filling proteins encoding genes, globulin (Os05g0499100) and prolamin (Os12g0269200) were designed using Primer 3 (Table 2.2) based on the genome sequence of rice in National Center for Biotechnology Information (NCBI) gene bank. The primer sets for actin, pathogenesis-related proteins encoding genes, *PR1b*, and *Pi21* genes were obtained from Fukuoka et al., (2009) (Table 2.2). *Pi21* acts as a negative regulator for rice blast disease resistance (Fukuoka et al., 2009).

Similarly, primers for real time PCR (qPCR) were prepared by using the program Beacon Designer (Premier Biosoft International, Palo Alto, CA) (Table 2.3) based on the rice genome sequence information available in the NCBI.

100 µM of those primers obtained from the Bioneer Inc. (Alameda, CA) were diluted to 10 µM to use as a working solution and stored at -20 °C.

Table 2.1. Bacterial strains used in this study.

Strain	Characteristics	Source
Burkholderia glumae		
336gr-1	Reference strain, wild type	This study
toxA$^-$	Toxoflavin production deficient mutant; Km^r	Dr. Nandakumar
toxA$^-$/*hrp*$^-$	Toxoflavin production and hypersensitive response deficient mutant; Km^r	Dr. Nandakumar
Rhizoctonia solani		
LR-71	Virulent strain	Dr. Rush

Table 2.2. Primers for reverse transcription PCR assays for the detection of induction of various defense related genes, pathogenesis related proteins, grain filling proteins in response to *B. glumae* and its mutant derivatives in Jupiter and Trenasse.

ID	Sequence (5'→3')	PCR Product (bp)	Tm (°C)	GC%	Source or reference
ActinF	TCCATCTTGGCATCTCTCAG	335	52.2	50	(Fukuoka et al., 2009)
ActinR	GTACCCGCATCAGGCATCTG		57.5	60	(Fukuoka et al., 2009)
Pi21F	CGGCAAATTTGACAGATGGGTAT	177	31.4	43.5	(Fukuoka et al., 2009)
Pi21R	CTTCTCCGGGTCGAACTTC		53.3	57.9	(Fukuoka et al., 2009)
PR1bF	GTTATTTATACACACGGGCGTA	217	60.7	39.3	(Fukuoka et al., 2009)
PR1bR	AACTTTAACCAAAGTTAATAGGT		61.1	42.9	(Fukuoka et al., 2009)
Globulin-F	GGAGATGAGGTTCAGGGACA	227	53.4	55	This study
Globulin-R	CCTCGTAGCTCCTCACCATC		53.4	60	This study
Prolamin-F	GCAGCACAGTGGCAACCCCC	292	64.3	70	This study
Prolamin-R	CCGACGGTGGGAATGCTACAGG		64.1	63.6	This study
NAC-likeF	CCTGACCTGCCTCCGGGCTT	362	64.2	70	This study
NAC-likeR	TTGTCGCCCTTGGGAGCCCT		64.5	65	This study
NAC4 F1	CCTCTGCCGCAAGGTTGCCC	392	65.5	70	This study
NAC4 R1	GCACCCACTCGTCCAGCTTC		59.1	65	This study

Table 2.3. Primers for SYBR Green PCR assay for the quantification of the induction of Os01g0393100 in response to *B. glumae* and its mutant derivatives in Jupiter and Trenasse.

ID	Sequence (5'→3')	PCR Product (bp)	Tm (°C)	GC%	Source or reference
NAC-like_RT_F	GCAGATGTTGGACGACTTC	77	49.7	52.6	This study
NAC-like_RT_R	CAGGTAGAGTGGAGTAGGAAG		47.1	52.4	This study
Actin_RT_F	GCCAATCGTGAGAAGATGAC	130	51.6	50	This study
Actin_RT_R	CACCAGAGTCCAACACATTAC		49	47.6	This study
18SrRNA F	ATGATAACTCGACGGATCGC	169	53.8	50	(Kim et al., 2003)
18SrRNA R	CTTGGATGTGGTAGCCGTTT		53.8	50	(Kim et al., 2003)

Table 2.4. Primers used for sequencing Os01g0393100 of Jupiter and Trenasse.

ID	Sequence (5'→3')	PCR Product (bp)	Tm (°C)	GC%	Source or reference
NAC-like_ORF_F	GCGAGACAATTAGGGAAGCATGCAA	1893	64	48	This study
NAC-like_ORF_R	AGCCAAAGGCAATGCAAAAGCCA		65.2	47.8	This study
NAC-like_Int_F	CGCTTCTACATTGGGGTGCTTGTTA	900	62	48	This study
NAC-like_Int_R	CAACCGGCACCGGCTTCTTGA		65.8	61.9	This study

2.1.6 Reverse Transcription (RT) PCR

The extracted total RNA was used to synthesize cDNA using the protocol from ProtoScript® M-MuLV First Strand cDNA Synthesis Kit, NEB #E6300S (New England BioLabs Inc., Ipswich, MA). Quantification of cDNA was done by using a Nano Drop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

The total of 25 µl of PCR reactions for the amplification was prepared as follows 16 µl of sterilized ddH_2O, 2.5 µl 10X PCR buffer containing 15 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 1 µl of *Taq* polymerase, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer and 3 µl of ~100 ng/µl template DNA. Amplification of the genes encoding for defensin, *NTF,* globulin, prolamin, *PR1b, Pi21* were done by using the primers listed in Table 2.2, and PCR condition: initial denaturation at 94 °C for 1 min., annealing at 55 °C for 30 sec, extension at 72 °C for 30 sec, amplification for 34 cycles and the final extension at 72 °C for 10 min using BIO-RAD DNAEngine® Peltier Thermal Cycler. The expression of actin was used to standardize the total RNA sample of each RT-PCR.

2.1.7 Agarose Gel Electrophoresis

The PCR products were run in a 1% Agarose gel (Amresco, Code-0710-100G) at 100 V for 1 hour followed by observation under KODAK Gel Logic 1500 Imaging System, Molecular Imaging Systems, Carestream Health, Inc. Rochester, NY 14608.

2.1.8 Quantification of Induced Gene Expression by Quantitative Real-Time PCR (qPCR)

Quantitative real-time RT PCR was performed in the iQ^{TM} 5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) with 10 µM of the gene-specific primers for the NAC-like transcription factor (Table 2.3). Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) was used to detect the expression of this gene in the RT-PCR products of two cultivars Jupiter and Trenasse treated under different treatments. 10 µM of the primers for 18S rRNA gene was used as an internal control gene, to normalize quantity of the expression, which has uniform expression under different treatments. The 20 µl reaction of mixture consisted of 10 µl of 2X Power SYBR Green Master Mix, 1 µl each of forward and reverse primers (10 µM), 2 µl of ~2ng/µl of cDNA template and 6 µl of nuclease free water.

Relative quantification method is used to analyze the change in the expression of target gene (*NTF*) by normalizing with the internal control gene 18S rRNA. Relative quantification expresses the relative fold changes in expression of the target gene in comparison to reference group. In this experiment, the non-inoculated panicles of both Jupiter and Trenasse were used as a reference group. Threshold cycle (C_t) value is used to quantify the relative changes in gene expression from which mean fold changes ($2^{-\Delta\Delta Ct}$) were calculated (Livak and Schmittgen, 2001). C_t cycle is the cycle at which the significant increase in the magnitude of fluorescence is detected. The experiment was repeated two times with three replications.

2.1.9 PCR Amplification and Sequence Analysis of Os01g0393100

Primers NAC like ORF_F and NAC like ORF_R (Table 2.4) were used to amplify the gene encoding NTF and its promoter regions of both Jupiter and Trenasse. At least three independent PCR reactions were conducted, and the PCR products were purified using the QuickClean 5M PCR Purification Kit (GenScript, Piscataway, NJ). Purified PCR products, from each independent reaction, were sequenced at the Louisiana State University School of Veterinary Medicine's GeneLab, Baton Rouge, LA, to ensure correct base identification.

NTF sequences from both rice cultivars were analyzed with the alignment of sequences with ClustalW2 (European Molecular Biology Laboratory's European Bioinformatics Institute, available at www.ebi.ac.uk (Chenna et al., 2003).

2.2 RESULTS

2.2.1 Disease Severity

Symptoms caused by *B. glumae* 336gr-1, and its derivatives *tox*$^-$, and *tox*$^-$/*hrp*$^-$, on the Jupiter and the Trenasse, were scored, with standard scale (0-9), from day 0 until one week. The disease severity was calculated as $\sum$ (number of samples with each rating × rating value)/total number of panicles (Devescovi et al., 2007). BPB symptoms in both cultivars sprayed with the bacterial strains were not appeared until DAI 2. Water spray on panicles of both cultivars was considered as a negative control, which did not produce any symptoms (Fig. 2.2), and also did not have any disease severity score (Fig. 2.2). Disease severity was scored in the panicles of both Jupiter and Trenasse, inoculated with bacteria from 4 days after inoculation (DAI) (Fig. 2.2). The disease severity is in increasing order over time as potted in a bar diagram (Fig. 2.2). Panicles of the cultivar Trenasse inoculated with *B. glumae* 336gr-1, toxoflavin deficient, and toxoflavin and HR deficient mutants of *B. glumae* showed higher disease severity than the Jupiter inoculated

with the same inocula (Fig. 2.2, and 2.3). In addition to that, in Trenasse, panicles inoculated with *B. glumae* 336gr-1 showed more disease severity than the panicles inoculated with toxoflavin deficient, or toxoflavin and HR deficient mutant derivatives of the *B. glumae* 336gr-1.

However, panicles inoculated with toxoflavin deficient mutant have higher disease severity than the panicles inoculated with toxoflavin and HR deficient mutant in both cultivars, Jupiter and Trenasse. However, Trenasse shows susceptibility to toxoflavin, and toxoflavin and HR deficient mutants by producing symptoms in the inoculated panicles (Fig. 2.3 c).

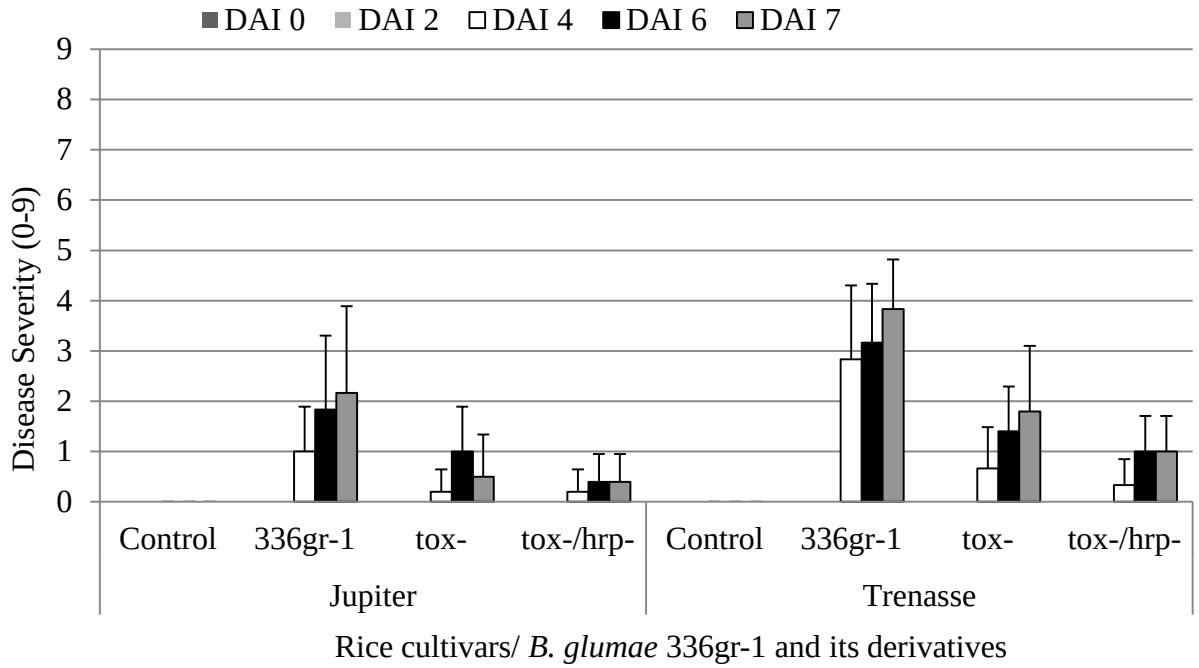

Figure 2.2 Disease rating on the panicles of Jupiter and Trenasse 0, 2, 4, 6 and 7 days after inoculation (DAI) of *B. glumae* 336gr-1, *B. glumae tox*$^{-}$ and *B. glumae tox*$^{-}$/*hrp*$^{-}$. 1×10^{8} CFU/ml of bacterial inoculum was sprayed. Water spraying was used as control. Disease scoring was done using standard scale (0-9). Similar pattern of scoring was obtained in two repeated experiments. Each error bar indicates standard error from two replicates.

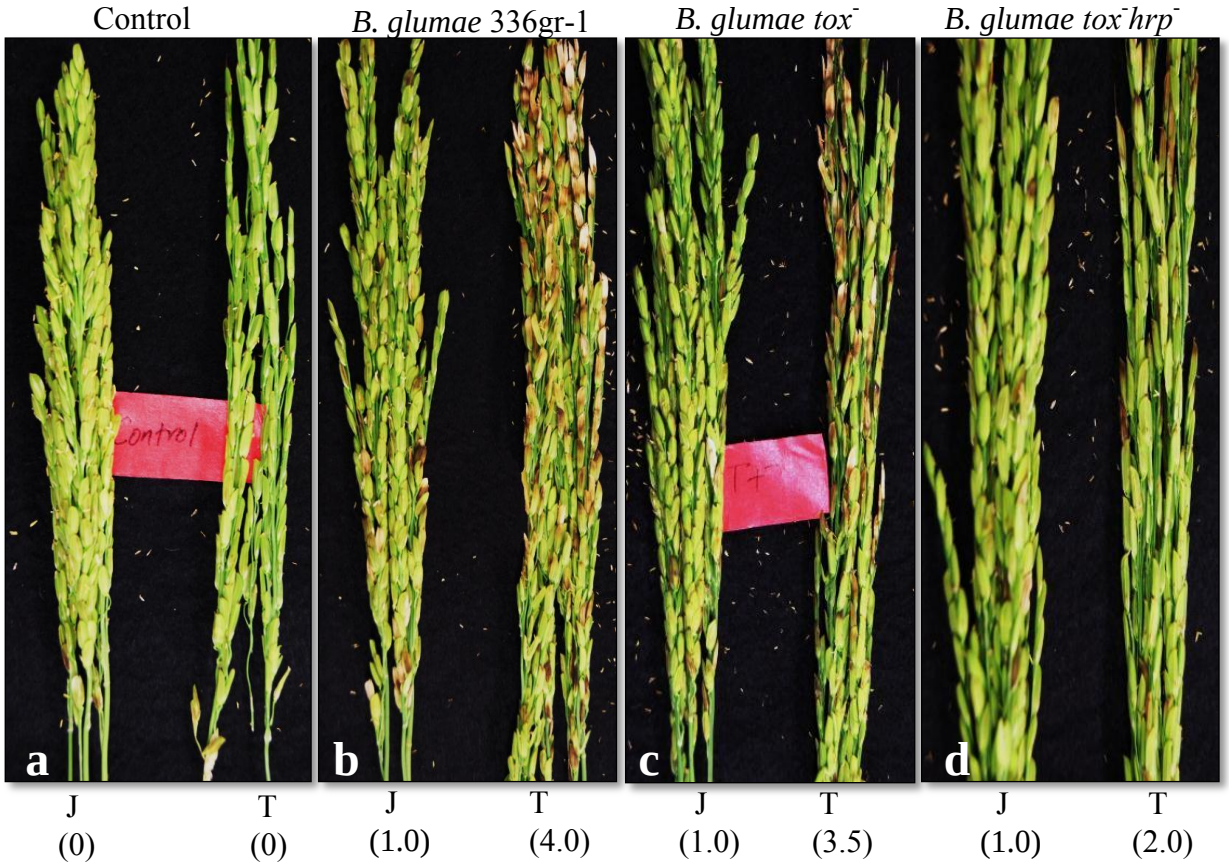

Figure 2.3 Bacterial panicle blight symptoms produced on the panicles of Jupiter and Trenasse by: a) water as a control; b) *B. glumae* 336gr-1; c) *B. glumae tox*$^{-}$, toxoflavin deficient mutants; and d) *B. glumae tox*$^{-}$*hrp*$^{-}$, toxoflavin and HR deficient mutants after 8 days. Panicle discoloration with unfilled grains is the severe symptoms caused by BPB. J= panicles from Jupiter, T=panicles from Trenasse, and numbers in the parenthesis denote the disease score.

2.2.2 Reverse Transcription (RT) PCR

RT-PCR was conducted by using the cDNA, as a template, prepared from the total RNA extracted from the panicles of the Jupiter and Trenasse inoculated with *B. glumae* 336gr-1 and its derivatives or water to see the induction of the expression of various genes, *Pi21*, *PR1b*, *NTF* (Os01g0393100), prolamin (Os12g0269200), NAC4 (Os01g0816100) and actin (Fig 2.4) that are involved in rice defense against BPB. Amongst all, *Pi21* (177 bp), NAC 4 (Os01g0816100) (392 bp) are induced uniformly in both Jupiter and Trenasse under all treatment conditions. However, *PR1b* was induced in Jupiter in all treatments, while, in Trenasse, it is only induced after the inoculation of toxoflavin production deficient, and toxoflavin and HR deficient mutant (Fig. 2.4. lane 3 and 4). Furthermore, Os12g0269200 is induced in Jupiter after the inoculation of *B. glumae* and its derivatives, but not in water treated panicles (Fig. 2.4. lane 1). However, in Trenasse, it is induced only in the panicles inoculated with toxoflavin and HR deficient mutant of *B. glumae*. Similarly, Os01g0393100 showed induction in Jupiter only after the inoculation of *B. glumae* and its derivatives (Fig. 2.4. Os01g0393100 row, lane 2, 3, and 4, Jupiter). In contrast, in Trenasse, this gene did not show any induction under any treatments (Fig. 2.4.NAC like row lane 2, 3, and 4, Trenasse). However, actin, with PCR product of 335 bp, was induced uniformly either in water sprayed control or bacterial inoculated panicles in both cultivars (Fig. 2.4, actin row). Genomic DNA of both Jupiter and Trenasse were used as a positive control, which is amplified in both cultivars.

Furthermore, Os01g0393100 is differentially expressed in each of the different treatments (Fig. 2.4). The *NTF* gene is induced more in the panicles of Jupiter after the inoculation with *B. glumae tox*$^{-}$ than the panicles inoculated with *B. glumae* 336gr-1 and *B. glumae tox*$^{-}$*/hrp*$^{-}$.

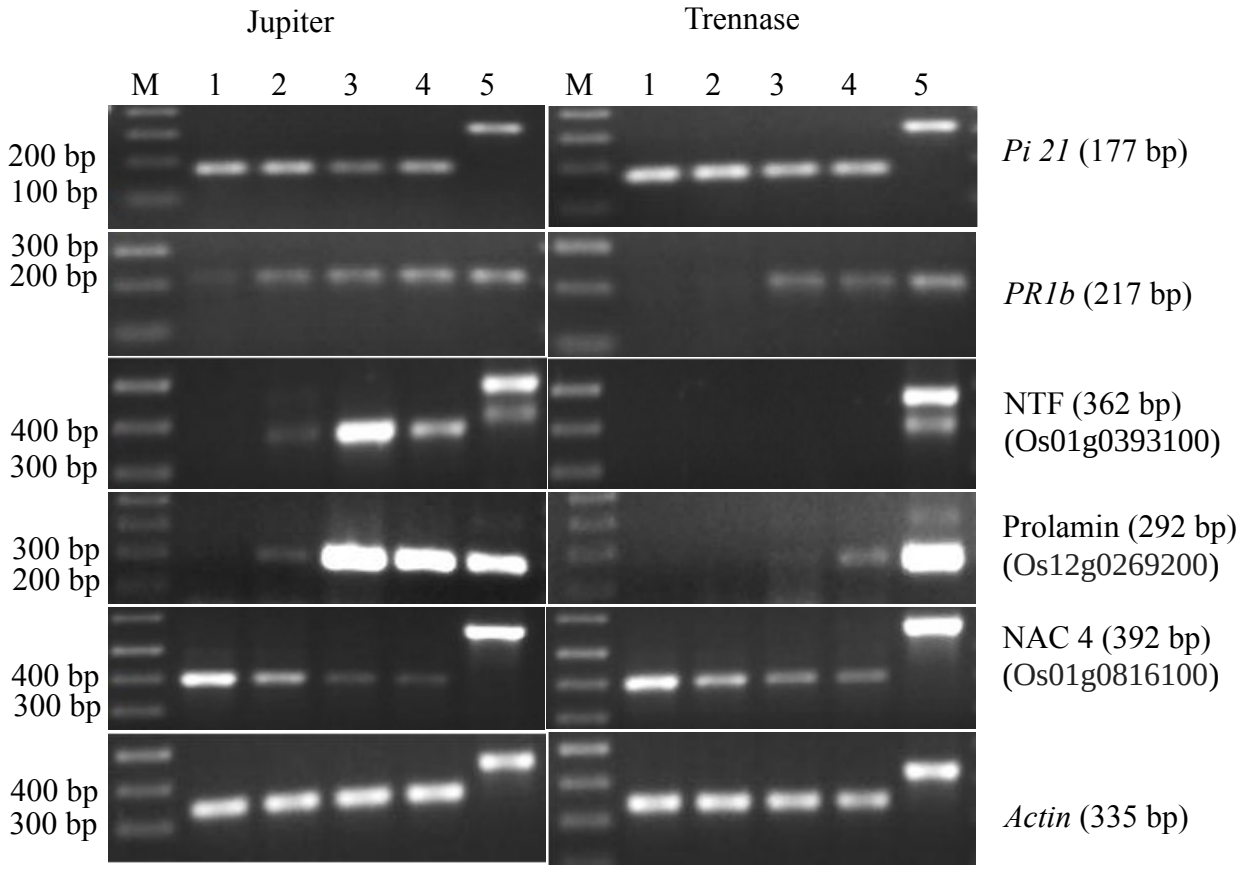

Figure 2.4 Expression patterns of Os01g0393100 and Os12g0269200 in Jupiter and Trenasse after inoculation with *B. glumae* and its derivatives or water (control) inoculation, analyzed by RT-PCR. M= 1 kb plus DNA ladder, lane 1= cDNA samples from water treated rice panicles, lane 2= cDNA from *B. glumae* 336gr-1 treated panicles, lane 3= cDNA from *B. glumae tox*$^-$ treated panicles, lane 4= cDNA from *B. glumae tox*$^-$ *hrp*$^-$ treated panicles, lane 5= genomic DNA from Jupiter and Trenasse used as a positive control.

2.2.3 Quantification of Induced Gene Expression by Real-Time PCR (qPCR)

The C_t values obtained from the qPCR were analyzed to observe the change in fold expression of the *NTF* gene. The fold change in expression of the *NTF* normalized to 18S rRNA was observed under various treatments on two different cultivars of rice. The data were analyzed by the formula $\Delta\Delta C_t = (\Delta C_t - \Delta \text{Average } C_t)$; where, $\Delta C_t = (C_{t,\,NTF} - C_{t,\,18S\ rRNA})$ under different treatments, and $\Delta \text{Average } C_t = (C_{t,\,NTF} - C_{t,\,18S\ rRNA})$ control (Livak and Schmittgen, 2001). The

mean fold change in *NTF* expression at control condition was very close to 1, i.e., 1.04 and 1.001, in both rice cultivar, Jupiter and Trenasse respectively (Table 2.5 and Table 2.7), which suggests that there is absence of high degree of experimental variation.

Mean fold change in gene expression was plotted in graph (Fig. 2.5), where there is mean fold changes of *NTF* in y-axis, and different treatments on Jupiter and Trenasse are in the x-axis. Inoculation of the *B. glumae* 336gr-1, *B. glumae tox*$^-$ and *B. glumae tox*$^-$*/hrp*$^-$ change the expression of *NTF* in Jupiter by 1.79, 69.97, and 12.84 fold respectively, whereas in Trenasse by 0.25, 0.24, and 0.34 fold respectively (Fig. 2.5).

Interestingly, *NTF* gene in Jupiter is differentially expressed after the bacterial inoculation. Panicles of Jupiter inoculated with the *B. glumae tox*$^-$ shows higher fold change in expression than the other panicles inoculated with *B. glumae* 336gr-1 and *B. glumae tox*$^-$*/hrp*$^-$ (Fig. 2.5).

Table 2.5 Data analysis for the relative quantification of target gene (*NTF*) by using $2^{-\Delta\Delta Ct}$ method. The relative fold change in expression of *NTF* gene in comparison to the endogenous control gene (18S rRNA) under three different treatments by *B. glumae* 336gr-1 and its mutant derivatives *B. glumae tox*$^-$ and *B. glumae tox*$^-$*hrp*$^-$ in BPB partial resistant cultivar, Jupiter.

Treatments	Primer	C_t value	Mean C_t control	$2^{-\Delta\Delta Ct}$	Mean fold change in gene expression	Standard Deviation
Control	NTF	34.32	34.62	1.079228237	1.047809023	0.373338
Control	NTF	34.82	34.62	1.404444876		
Control	NTF	34.72	34.62	0.659753955		
B. glumae 336gr-1	NTF	33.53	34.62	2.0139111	1.791539648	0.314481
B. glumae 336gr-1	NTF	34.21	34.62	1.569168196		
B. glumae 336gr-1	NTF		34.62			
B. glumae tox$^-$	NTF	27.81	34.62	81.00842201	69.97037259	9.600891
B. glumae tox$^-$	NTF	27.81	34.62	65.34477605		
B. glumae tox$^-$	NTF	27.65	34.62	63.55791971		
B. glumae tox$^-$*hrp*$^-$	NTF	31.77	34.62	17.63048185	12.84480107	4.157315
B. glumae tox$^-$*hrp*$^-$	NTF	31.59	34.62	10.77786861		
B. glumae tox$^-$*hrp*$^-$	NTF	31.85	34.62	10.12605275		

Table 2.6 The relative fold change in expression of 18S rRNA under three different treatments by *B. glumae* 336gr-1 and its mutant derivatives *B. glumae tox*$^-$ and *B. glumae tox*$^-$*hrp*$^-$ in BPB partial resistance cultivar, Jupiter.

Treatments	**Primer**	**C_t value**	**Mean C_t control**
Control	18S rRNA	18.51	18.7
Control	18S rRNA	19.39	18.7
Control	18S rRNA	18.2	18.7
B. glumae 336gr-1	18S rRNA	18.62	18.7
B. glumae 336gr-1	18S rRNA	18.94	18.7
B. glumae 336gr-1	18S rRNA	18.68	18.7
B. glumae tox$^-$	18S rRNA	18.23	18.7
B. glumae tox$^-$	18S rRNA	17.92	18.7
B. glumae tox$^-$	18S rRNA	17.72	18.7
B. glumae tox$^-$*hrp*$^-$	18S rRNA	19.99	18.7
B. glumae tox$^-$*hrp*$^-$	18S rRNA	19.1	18.7
B. glumae tox$^-$*hrp*$^-$	18S rRNA	19.27	18.7

Table 2.7 Data analysis for the relative quantification of target gene (*NTF*) by using $2^{-\Delta\Delta Ct}$ (mean fold change) method. The relative fold change in expression of *NTF* gene in comparison to the endogenous control gene (18S rRNA) under three different treatments by *B. glumae* 336gr-1 and its mutant derivatives *B. glumae tox*$^-$ and *B. glumae tox*$^-$*hrp*$^-$ in BPB susceptible cultivar, Trenasse.

Treatments	**Primer**	**C_t value**	**Mean C_t control**	**$2^{-\Delta\Delta Ct}$**	**Mean fold change in gene expression**	**Standard Deviation**
Control	NTF	36.88	37.06333333	1.028113827	1.001472857	0.065745
Control	NTF	37.32	37.06333333	1.049716684		
Control	NTF	36.99	37.06333333	0.926588062		
B. glumae 336gr-1	NTF	36.63	37.06333333	0.373712312	0.252821771	0.12497
B. glumae 336gr-1	NTF	37.42	37.06333333	0.26061644		
B. glumae 336gr-1	NTF	38.08	37.06333333	0.124136562		
B. glumae tox$^-$	NTF	37.52	37.06333333	0.283220971	0.245663817	0.032563
B. glumae tox$^-$	NTF	37.65	37.06333333	0.225312616		
B. glumae tox$^-$	NTF	37.9	37.06333333	0.228457863		
B. glumae tox$^-$*hrp*$^-$	NTF	37.02	37.06333333	0.473028823	0.341493593	0.116782
B. glumae tox$^-$*hrp*$^-$	NTF	37.13	37.06333333	0.301451957		
B. glumae tox$^-$*hrp*$^-$	NTF	37.27	37.06333333	0.25		

Table 2.8 The relative fold change in expression of 18S rRNA under three different treatments by *B. glumae* 336gr-1 and its mutant derivatives *B. glumae tox*$^-$ and *B. glumae tox*$^-$*hrp*$^-$ in BPB partial resistance cultivar, Trenasse.

Treatments	**Primer**	**C_t value**	**Mean C_t control**
Control	18S rRNA	31.12	31.26333333
Control	18S rRNA	31.59	31.26333333
Control	18S rRNA	31.08	31.26333333
B. glumae 336gr-1	18S rRNA	29.41	31.26333333
B. glumae 336gr-1	18S rRNA	29.68	31.26333333
B. glumae 336gr-1	18S rRNA	29.27	31.26333333
B. glumae tox$^-$	18S rRNA	29.9	31.26333333
B. glumae tox$^-$	18S rRNA	29.7	31.26333333
B. glumae tox$^-$	18S rRNA	29.97	31.26333333
B. glumae tox$^-$*hrp*$^-$	18S rRNA	30.14	31.26333333
B. glumae tox$^-$*hrp*$^-$	18S rRNA	29.6	31.26333333
B. glumae tox$^-$*hrp*$^-$	18S rRNA	29.47	31.26333333

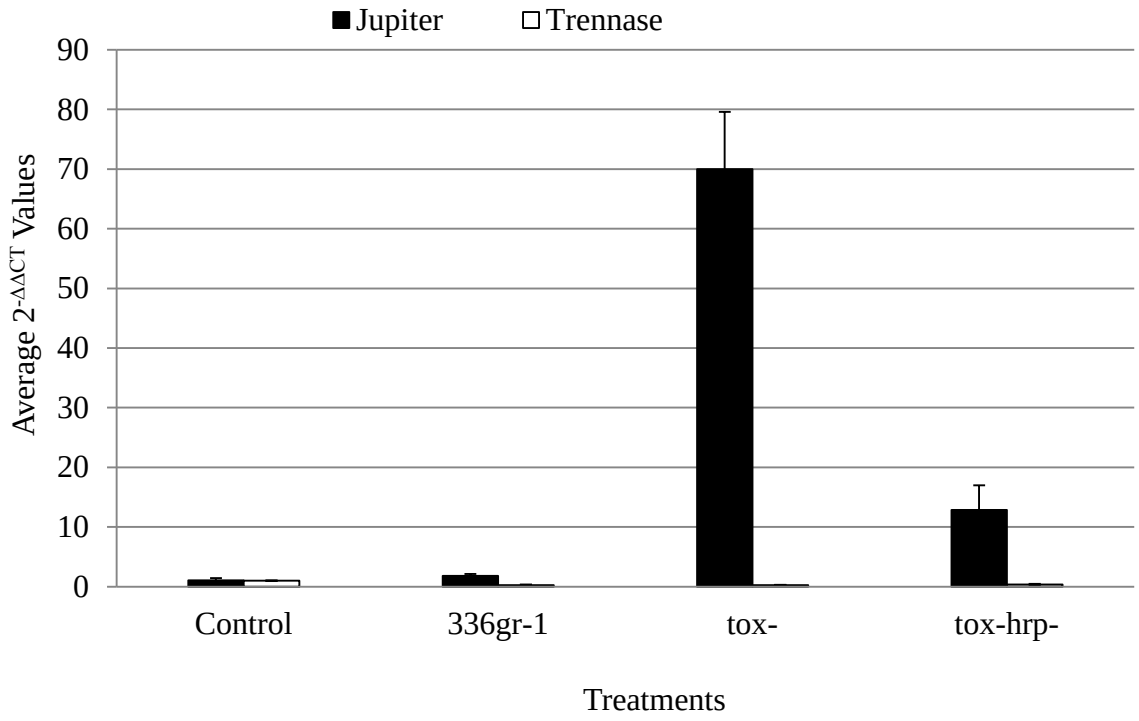

Figure 2.5 Differential $2^{-\Delta\Delta Ct}$ values under different treatments in Jupiter and Trenasse. Expression of gene encoding Os01g0393100 in response to different treatments: water control, *B. glumae* 336gr-1, *B. glumae tox*$^{-}$, *B. glumae tox*$^{-}$*/hrp*$^{-}$. Solid and empty bar denotes the change in the expression of *NTF* in Jupiter and Trenasse respectively. Similar pattern of fold changes in expression was observed in repeated experiments. Each error bar indicates standard error from three replicates.

2.2.4 Sequence Analysis of Os01g0393100 (*NTF*) between Jupiter and Trenasse

Sequences of Os01g0393100 (*NTF* gene) in rice were obtained from both Jupiter and Trenasse (Appendix C). ClustalW2 alignment of this sequence showed 100% identical between both Jupiter and Trenasse (Appendix D).

3. ALTERNATIVE METHODS TO SUPPRESS THE SYMPTOMS OF BPB IN RICE, AND THE GROWTH OF *BURKHOLDERIA GLUMAE* AND *RHIZOCTONIA SOLANI IN VITRO*

3.1 MATERIALS AND METHODS

3.1.1 Plant Materials

3.1.1.1 Field Experiments

The rice cultivar used in this experiment was the long-grained, susceptible cultivar Trenasse, grown in the field at the Rice Research Station, Crowley, LA in summer, 2010. There were approximately15 hills of rice plants in a row for each treatment. There were 21 rows of rice plants for eight different treatments with three different concentrations except for SA and water treatment with only two rows and one row respectively.

Similarly, we repeated the same experiment two times in the field in summer, 2011, using the susceptible rice cultivars Bengal and CL151. There were 81 rows of rice plants for nine treatments and three replications for Bengal whereas; there were 21 plot each containing 6 rows, for each concentration of the 6 treatments for CL151 for the second planting of rice.

Non-inoculated rows were used as negative control.

3.1.1.2 Greenhouse Experiments

In addition, we also conducted another set of experiment in the greenhouse in summer, 2011. We planted 19 pots of rice for seven different treatments with three different concentrations except for SA and water treatment with 2 pots and one pot respectively. Non-inoculated rice pots were used as a negative control.

3.1.2 Extraction of Culture Filtrate

Culture filtrate obtained from the King's B medium after growing the *B. glumae* for 48 hours was used as an elicitor. Culture filtrate contained yellow-pigmented phytotoxin toxoflavin (Appendix B). Toxoflavin acts as an effective electron carrier and helps to generate peroxides (Latuasan and Berends, 1961). Extraction of CF was done according to the procedures developed by Iiyama et al., 1995, with few modifications. *B. glumae* 336gr-1 was streaked in King's B medium and incubated at 37 °C for 48 hours. The bacterial cells on the media were washed off with sterilized cotton swab and washed with sterilized ddH_2O. The remaining agar was cut into pieces with the help of spatulas and weighed. The CF was extracted by adding an equal amount of chloroform (weight/volume) in the agar medium. The chloroform was evaporated and the residues were dissolved in 1 ml of aqueous 80% methanol. The dissolved CF was again diluted in sterilized ddH_2O as per required in the experiments.

3.1.3 Pretreatment with Various Elicitors

After rice plants reached the 30% heading stages, various chemical compounds, which act as elicitors to induce SAR in plants, were used for the pretreatment. Each of the chemical (Table 3.1) and material including culture filtrate was pretreated 24 hours before the inoculation of *B. glumae* 336gr-1 in three different concentrations. These elicitors were identified to induce defense related genes in rice. The elicitors along with their concentrations that were used in field experiment of summer 2010, and 2011 and greenhouse experiment of summer 2011 are as follows:

Table 3.1 Elicitors and their three different concentrations used for the pretreatment of Trenasse in the field in summer 2010 and in the greenhouse in summer 2011.

S.No.	Elicitors	Concentrations used
1.	Ethephon (ET)[a]	50 µM, 100 µM, and 200 µM.
2.	Jasmonic acid (JA)	50 µM, 100 µM, and 200 µM.
3.	Salicylic acid (SA)	50 µM, 100 µM, and 200 µM.
4.	2, 6-Dichloroisonicotinic acid (INA)	50 µM, 100 µM, and 200 µM.
5.	Ascorbic acid (AA)	50 µM, 100 µM, and 200 µM.
6.	Hydrogen peroxide (H_2O_2)	50 µM, 100 µM, and 200 µM.
7.	Culture filtrate (CF)	10 times, 20 times and 100 times dilution of the extracted culture filtrate.

[a]Ethephon was not included in greenhouse experiment in 2011.

Table 3.2 Elicitors and their three different concentrations used for the pretreatment of 1st planting of Bengal and 2nd planting of CL151 in the field in summer 2011.

S.No.	Elicitors	Concentrations used
1.	Jasmonic acid (JA)	10 µM, 50 µM, and 100 µM.
2.	Salicylic acid (SA)[a]	100 µM, 200 µM, and 500 µM.
3.	2, 6-Dichloroisonicotinic acid (INA)	100 µM, 200 µM, and 500 µM.
4.	Ascorbic acid (AA)[b]	50 µM, 100 µM, and 200 µM.
5.	β-amino butyric acid (BABA)[c]	50 µM, 100 µM, and 200 µM.
6.	Citric acid (CA)	50 µM, 100 µM, and 200 µM.
7.	Hydrogen peroxide (H_2O_2)[d]	0.5 µM,5 µM, and 50 µM.
8.	Culture filtrate (CF)[e]	10 times, 100 times, 1000 times, and 10000 times dilution of the extracted culture filtrate.

[a, b, c,] and [d] Elicitors repeated in 2nd planting in the field 2011.

[e] Used only 100 times dilution in 2nd planting in the field.

3.1.4 Inoculation with *B. glumae*

3.1.4.1 Field Experiments

Pure culture of *B. glumae* 336gr-1 (Table 2.1) was obtained from a freshly grown single colony derived from glycerol stock. This pure culture of strain was streaked heavily on King's B media and incubated at 37 $^{\circ}$C one day prior to inoculation. About 100 ml of OD_{600}=0.1 (1×10^8 CFU/ml) of bacterial suspensions was prepared in sterile ddH_2O for each row, and inoculated 24 hours after the pretreatment of the various elicitors (Table 3.2). Inoculation was done with the help of hand sprayer in each row for the different concentrations of each treatment.

Sterile ddH_2O was applied as a negative control.

3.1.4.2 Greenhouse Experiments

For the greenhouse experiments, about 50 ml of 1×10^8 CFU/ml of bacterial suspension was prepared in sterile ddH_2O for each pot, and inoculated 24 hours after the pretreatment of the elicitors with three different concentrations (Table 3.1). Hand sprayers were used to inoculate the bacterial suspension in each pot, and kept separate until it dries to prevent cross contamination. Sterile ddH_2O was used as a negative control.

3.1.5 Disease Severity Assessment and Harvesting

Diseases symptoms were scored 10 days after inoculation (DAI) in the field and the greenhouse. In each treatment disease severity was scored by using standard scale of 0 to 9 (where 0 means no disease symptoms, 1 = 1 to 10% symptomatic grain on the panicles, 2 = 11 to 20% disease symptoms, 3 = 21 to 30% disease symptoms, 4 = 31 to 40% disease symptoms, 5 = 41 to 50% disease symptoms, 6 = 51 to 60% disease symptoms, 7 = 61 to 70% disease symptoms, 8 = 71 to 80% disease symptoms, 9 = more than 80% disease symptoms). Diseases

severity was calculated as ∑ (number of samples with each rating X rating value)/total number of panicles (Devescovi et al., 2007).

Rice plants were harvested, dried at room temperature, threshed and weighed. The comparison of yield loss was made between different concentrations of various elicitors treatments including non-inoculated rice plants.

3.1.6 Statistical Analysis

Disease severity data from both the greenhouse and the field were analyzed with Kruskal-Wallis analysis and post hoc test was done by using Dunn's test at significance level of 0.05. All statistical calculations were done with SAS procedure (SAS Institute, Inc. 2009).

3.1.7 Suppression of *B. glumae* and *R. solani in vitro* by The Antagonistic Effect of The Endophytes Isolated from Rice Leaves

3.1.7.1 Isolation of Endophytes from Rice Leaves

Leaves from the heading stage of several rice cultivars: CL-131, Catahoula, Cheniere, Neptune, Cocodrie and M2O1, were cut into pieces and washed either in a sterilized ddH_2O or in a solution of 10% bleach for 10 and 5 minutes, respectively. The leaf pieces were kept on a potato dextrose agar (PDA) medium making contact of upper part of leaf with the media. Three pieces of leaf were kept in one plate and incubated at room temperature for 3 days. The growth of several organisms; bacteria and fungi were observed around the contacted leaf pieces which are then transferred to the new PDA plates. After successive transfer of those bacteria and fungi for several times about 127 different isolates of bacterial endophytes were obtained from all cultivars of rice.

3.1.7.2 Antibacterial and Antifungal Assays

After isolation, those 127 isolates were examined for their antagonistic effect to *B. glumae* 336gr-1 and *Rhizoctonia solani* (LR-71) causing sheath blight of rice. Out of 127 isolates of endophytes, 29 isolates showed antagonistic activities to *B. glumae* or *R. solani.* Those 29 isolates were stored in at – 80 °C for further use. Further experiments were conducted for the confirmation of the antagonistic effect against both pathogens.

B. glumae 336gr-1was cultured overnight in LB broth at 37 °C. Overnight grown culture of *B. glumae* was centrifuged at 13000 rpm for 2 minutes. The supernatant was discarded and the pellet was resuspended in LB broth and centrifuged again for 2 minutes at 13000 rpm. The pellet was resuspended again in LB broth, after discarding the supernatant, to make 1×10^8CFU/ml of bacterial suspension (OD_{600}=0.1). 100 µl of 1×10^8CFU/ml of bacterial suspension of *B. glumae* was spread on the PDA and placed under a laminar flow hood until dried.

Simultaneously, each isolates of endophytes were also cultured in LB broth at 37 °C overnight, and 1.5 ml of each culture were centrifuged next day for 2 min at 13000 rpm. The supernatant was discarded and the pellet was resuspended in 1 ml of fresh LB broth. The resuspended solution was centrifuged at 13000 rpm for 2 minutes, and the supernatant was discarded. The pellet was then resuspended in 100 µl of LB broth. 10 µl of each of the isolates were spotted at three places for both antibacterial and antifungal assays. For antibacterial activity spotting was done over the *B. glumae* spreaded PDA plates, but for antifungal activity spotting was done only on media.

R. solani was grown from sclerotia on PDA plates and incubated at 30 °C for one week. One plug of approximately 5mm diameter of one week old *Rhizoctonia solani* (LR-71) was

placed at the center of the PDA media where the 10 µl of suspension of endophytes was spotted on the media.

The plates were kept in an incubation boxes and the boxes were wrapped with aluminium foil and incubated at 27 °C. Observations and measurements were taken after 3 days. This experiment was done in three replications for each of the endophytes.

3.2 RESULTS

3.2.1 Pretreatment of Elicitors

Two of the six chemical compounds, INA and AA, and the culture filtrate (CF), that were used for pretreatment in the field in 2010 show reduction in the symptoms of BPB in susceptible cultivar, Trenasse. Ascorbic acid with three different concentrations, 50 µM, 100 µM, and 200 µM helped to reduce the disease severity significantly with the score of 1.9, 1.8 and 1.2, respectively (Appendix H1). In contrast, same concentrations of hydrogen-peroxide (H_2O_2) and water pretreated panicles have significantly highest disease scores (Fig. 3.1 and Appendix H1) . Similarly, 100 µM of ET, JA, INA and SA also lowered the disease severity in Trenasse. BPB symptoms were not developed in the panicles with no inoculation.

The non-inoculated panicle row yielded higher than the inoculated panicle row. Rice yield from the rows pretreated with ET, JA, INA and H_2O_2 have lower yield than non-inoculated row (Fig.3.2). However, row pretreated with ascorbic acid has minimal yield reduction as compare to the other treatments and also water pretreated rows (Fig. 3.2).

Moreover, the experiment was repeated in greenhouse condition in summer 2011. In this experiment, non-inoculated panicles did not show any BPB symptoms, in contrast panicles pretreated with 1/10X CF have highest disease severity of 5.0 (Fig. 3.3). Panicles pretreated with

JA, INA, H_2O_2, SA, and water did not show any significant difference in reducing the disease in rice plants (Fig. 3.3, Appendix H1). However, pretreatment of 100 µM and 200 µM of AA reduced the disease severity significantly as compared to other pretreatments (Appendix H1), which were 1.5 and 0.75 respectively (Fig. 3.3).

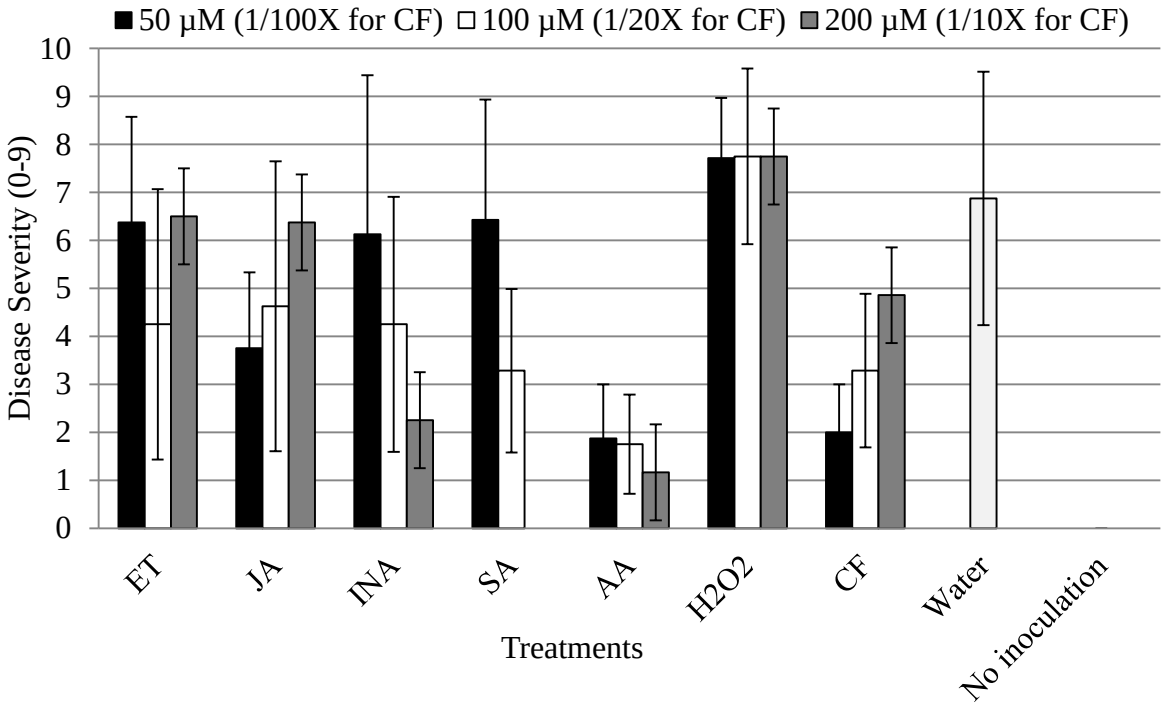

Figure 3.1 Ascorbic acid suppressed BPB symptoms in Trenasse (field experiment data in 2010). Disease rating on the panicles of Trenasse was done at 10 DAI of *B. glumae* 336gr-1. 1×10^8 CFU/ml of bacterial inoculum was inoculated 24 hours after the pretreatment of various elicitors each of with three different concentrations 50 µM, 100 µM and 200 µM. However, culture filtrate was diluted 10 times, 20 times and 100 times before pretreatment. Ethephon (ET), jasmonic acid (JA), 2, 6-Dichloroisonicotinic acid (INA), salicylic acid (SA), ascorbic acid (AA), hydrogen peroxide (H_2O_2), and culture filtrate (CF) were the elicitors used for pretreatment. Pretreatment with water was used as a positive control. No inoculation was used as a negative control. Disease scoring was done using standard scale (0-9) after 10 days. Effects of treatments were significantly different, $p= <0.0001$ from χ^2 test which is less than $\alpha=0.05$. Each error bar indicates standard error from five replicates.

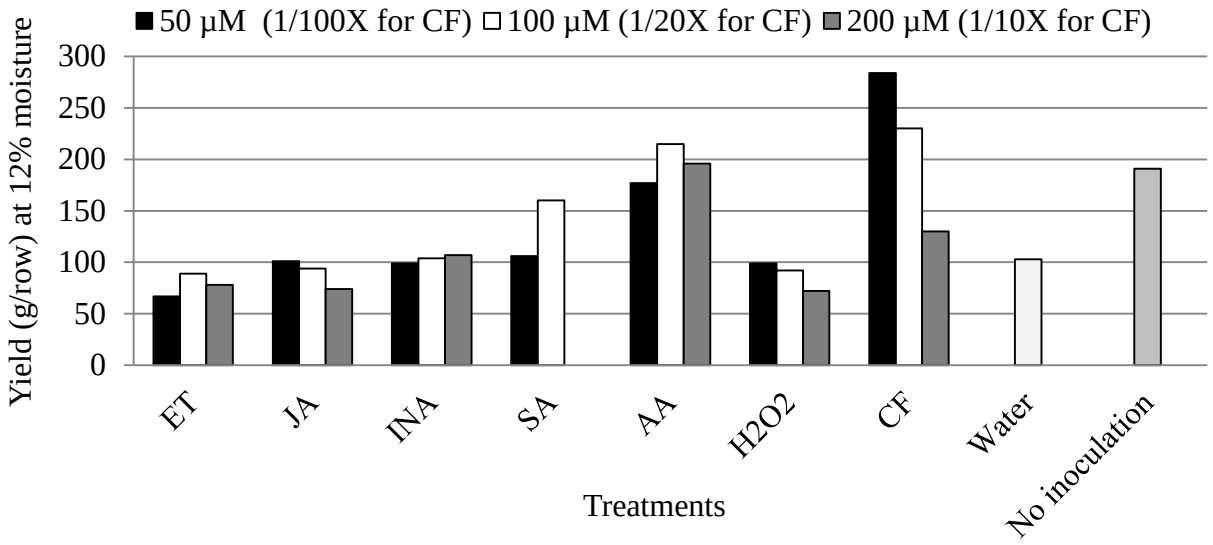

Figure 3.2 Ascorbic acid minimally reduced yield in Trenasse (field data in 2010). Rice yield (g/row) obtained from pretreated rice panicles by various elicitors each of with three different concentrations. Rice yield was weighed at 12% moisture condition. AA pretreated panicles has yield with minimal reduction; whereas water pretreated panicles have lower yield as compare to non-inoculated rows of rice.

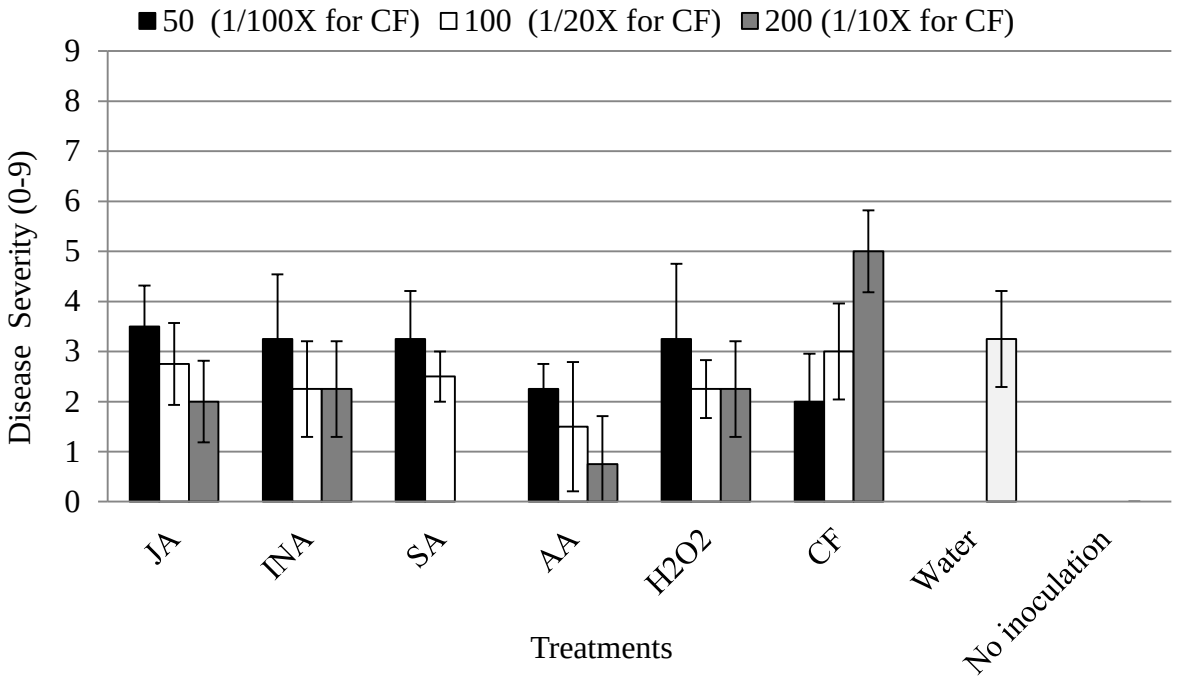

Figure 3.3 Ascorbic acid suppressed BPB symptoms in Trenasse (greenhouse data in 2011). Disease rating on the panicles of Trenasse was done at 10 DAI of *B. glumae* 336gr-1. 1×10^8 CFU/ml of bacterial inoculum was inoculated 24 hours after the pretreatment of various elicitors each of with three different concentrations 50 µM, 100 µM and 200 µM. However, culture filtrate was diluted 10 times, 20 times and 100 times before pretreatment. Jasmonic acid (JA), 2, 6-dichloroisonicotinic acid (INA), salicylic acid (SA), ascorbic acid (AA), hydrogen peroxide (H_2O_2), and culture filtrate (CF) were the elicitors used for pretreatment. Pretreatment with water was used as a positive control. No inoculation was used as a negative control. Disease scoring was done using standard scale (0-9) after 10 days. Effects of treatments were significantly different, p= 0.0012 from χ^2 test which is less than α=0.05.Each error bar indicates standard error from four replicates.

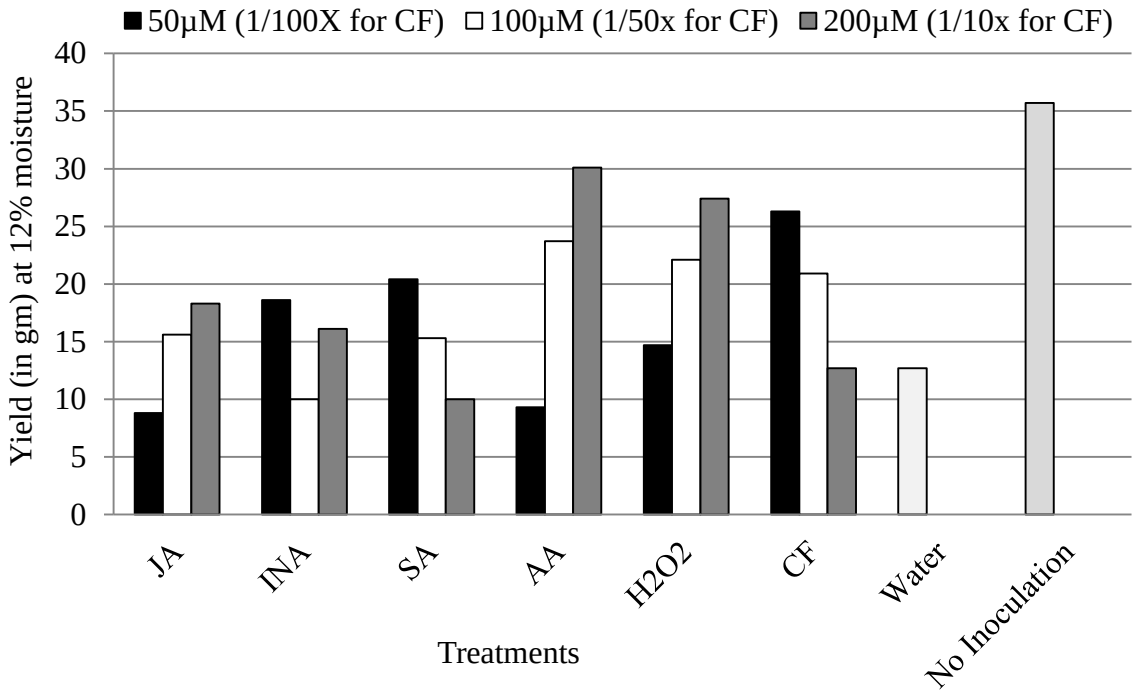

Figure 3.4 Ascorbic acid minimally reduced yield in Trenasse in the greenhouse in 2011. Rice yield (g/row) obtained from pretreated rice panicles by various elicitors each of with three different concentrations. Rice yield was weighed at 12% moisture condition. AA pretreated panicles has yield with minimal reduction; whereas water pretreated panicles have lower yield as compare to non-inoculated rows of rice with the highest yield.

After maturation, yield from rice plants pretreated with various chemicals were harvested and compared between the treatments. Non-inoculated rice plant had the highest yield of 35.7 g, and rice panicles pretreated with 100 µM and 200 µM of AA, which showed lowest disease severity, had minimal reduction in the yield compared to the non-inoculated rice panicles, however, higher yield than the panicles pretreated with water only (Fig.3.4). CF with 1/100 X and 1/50 X dilutions also showed higher yield than water pretreated panicles. Similarly, pretreatments of 100 µM and 200 µM of JA, 50 µM and 200 µM of INA, 50 µM and 100 µM of SA, and 100 µM and 200 µM of H_2O_2 resulted in higher yield than that of water only (Fig. 3.4), but these yields were lower than the yield from the panicles pretreated with the AA.

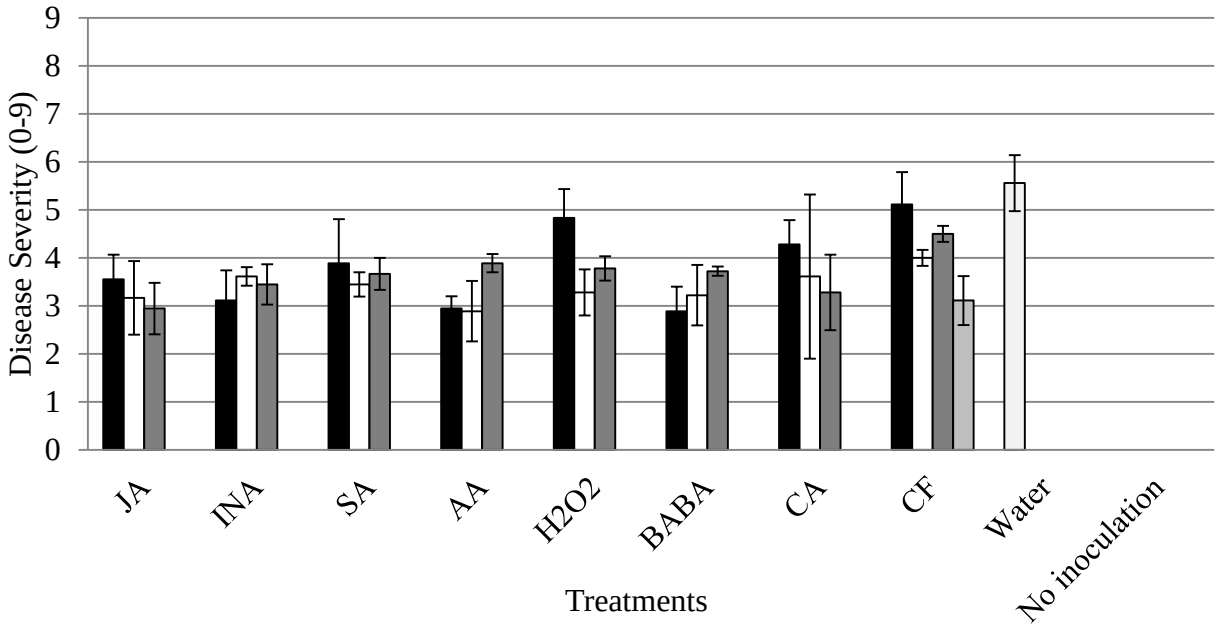

Figure 3.5 Ascorbic acid suppressed BPB symptoms in Bengal. 1st planting field data, 2011. Disease rating on the panicles of Bengal was done at 10 DAI of *B. glumae* 336gr-1. 1×10^8 CFU/ml of bacterial inoculum was inoculated 24 hours after the pretreatment of various elicitors each of with three different concentrations 10 µM, 50 µM, and 100 µM for JA; 100 µM, 200 µM, and 500 µM for INA; 100 µM, 200 µM, and 500 µM for SA; 50 µM, 100 µM, and 200 µM for AA and 200 µM; 0.5 µM, 5 µM, and 50 µM for H_2O_2; 50 µM, 100 µM, and 200 µM for BABA; 50 µM, 100 µM, and 200 µM for citric acid However, culture filtrate was diluted 10 times, 100 times, 1000 times and 10000 times before pretreatment. Jasmonic acid (JA), 2, 6-dichloroisonicotinic acid (INA), salicylic acid (SA), ascorbic acid (AA), hydrogen peroxide (H_2O_2), BABA, CA and culture filtrate (CF) were the elicitors used for pretreatment. Pretreatment with water was used as a positive control. No inoculation was used as a negative control. Disease scoring was done using standard scale (0-9) after 10 days. Each error bar indicates standard error from three replicates.

In addition to the previous elicitors, BABA and citric acid (CA) were included in the repeated field experiment for the second year in summer 2011. Disease symptoms were absence in the non-inoculated rows, whereas, maximum disease severity was scored in the row pretreated with water only. Pretreatment with various elicitors including BABA and CA helped to reduce the BPB symptoms on Bengal compared to the pretreatment with water only, but there is not any significant reduction in the disease symptoms (Appendix H1). However, pretreatment with

1/10000X dilution of CF showed more disease symptoms, but 1/10X dilution helped to reduce the symptoms (Fig. 3.5). The rows pretreated with AA showed lower disease severity score than those pretreated with other elicitors. Significant suppression of BPB was not observed after the pretreatment (Fig. 3.5, Appendix E1and H1).

Yield obtained was compared between the treatments in which rows pretreated with water had lower yield than other pretreated rows. Non-inoculated rows had higher yield, however, pretreatment of the elicitors did not show significant difference between the treatments (Fig. 6, Appendix E2 and H2). Pretreatment of INA with 200 µM had the highest yield of 343.9 g (Fig. 3.6).

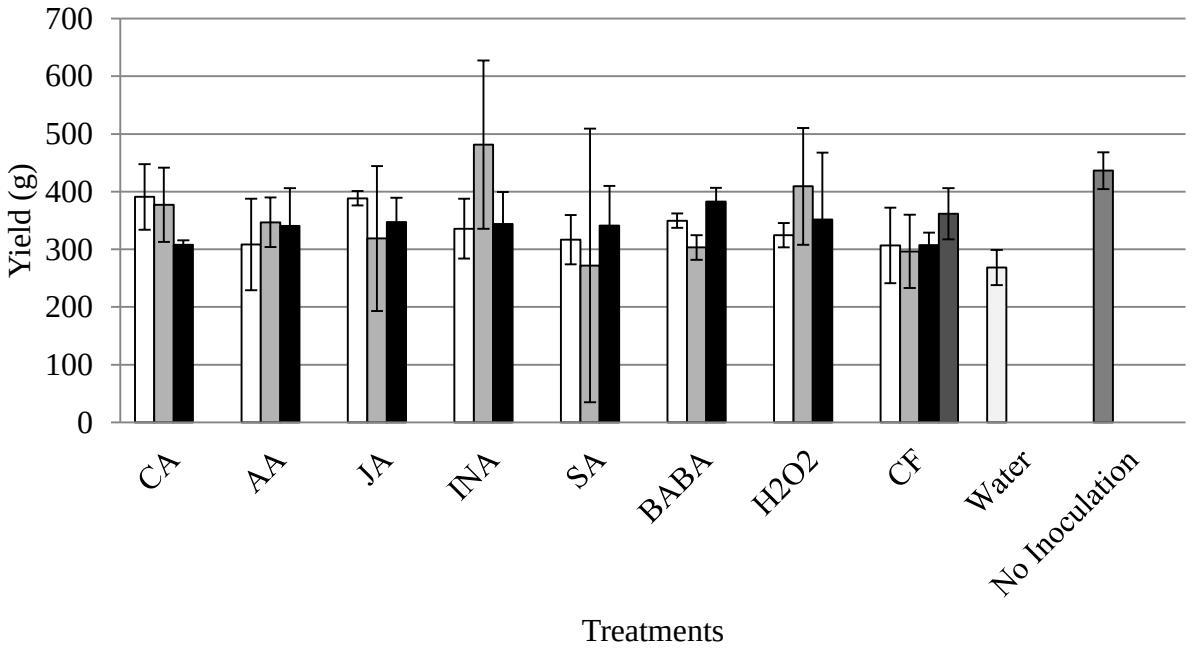

Figure 3.6.Rice yield (g/row) obtained from pretreated Bengal panicles (in 1st field planting, 2011) by various elicitors each of with three different concentrations. Rice yield was weighed at 12% moisture condition. Each error bar indicates standard error from three replicates.

3.2.2 Antibacterial and Antifungal Assays

Three days after inoculation *B. glumae* as well as *R. solani* was unable to cover the spots of several endophyptes and form an inhibition area (Fig. 3.9 and 3.10). Areas of the inhibition zone for both antibacterial and antifungal activities were calculated (Appendix F and G). There is a variation in the developed inhibition zone among the colonies. Isolates EP-1, EP-5, EP-12 and RCRIA1 did not show any antibacterial activity against *B. glumae*. EP-3 has the lowest activity of 1.34 cm^2 and EP-23S has the highest of 5.26 cm^2 of inhibition zone (Fig. 3.7 and Fig. 3.9).

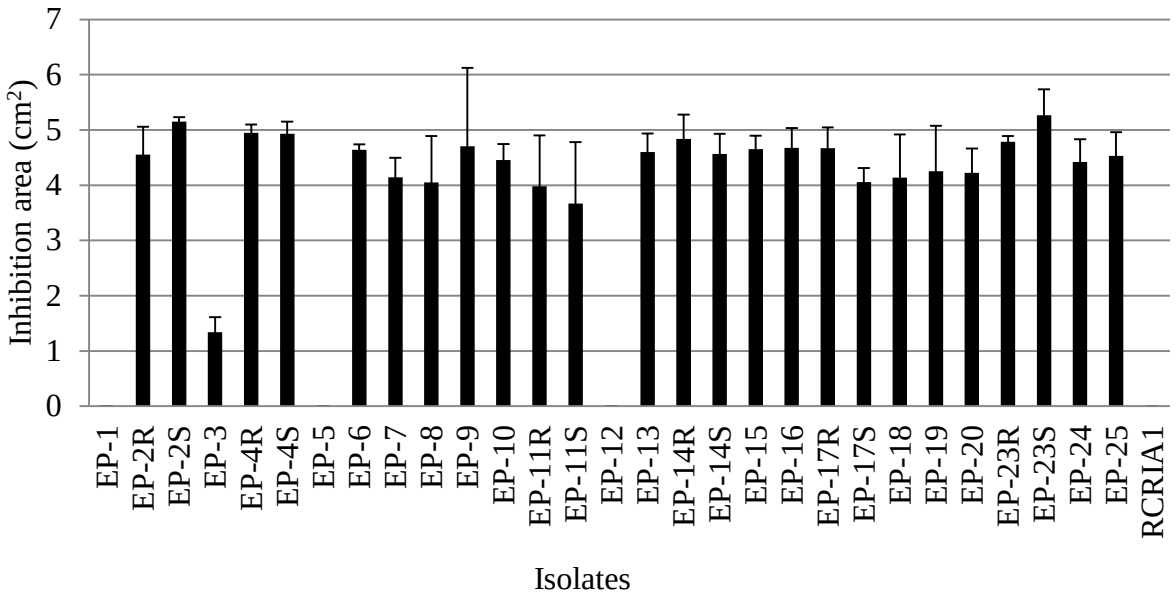

Figure 3.7 Antibacterial activities of the rice leaf endophytes against *B. glumae*. EP-1, EP5, EP-12 and RCRIA1 do not show antibacterial activity. EP-3 has the lowest activity and EP-23S has the highest inhibition area with 5.26 cm^2. Each error bar indicates standard error from three replicates.

Similarly, isolates EP-1, EP-5, EP-12 and RCRIA1 did not show any antifungal activity against *R. solani* (LR-71) (Fig. 3.8). EP-3, EP-8 and EP-17R have the lowest inhibition area showing low antifungal activities. There is a variation in the antifungal activities among the isolates (Fig. 3.8 and 3.10).

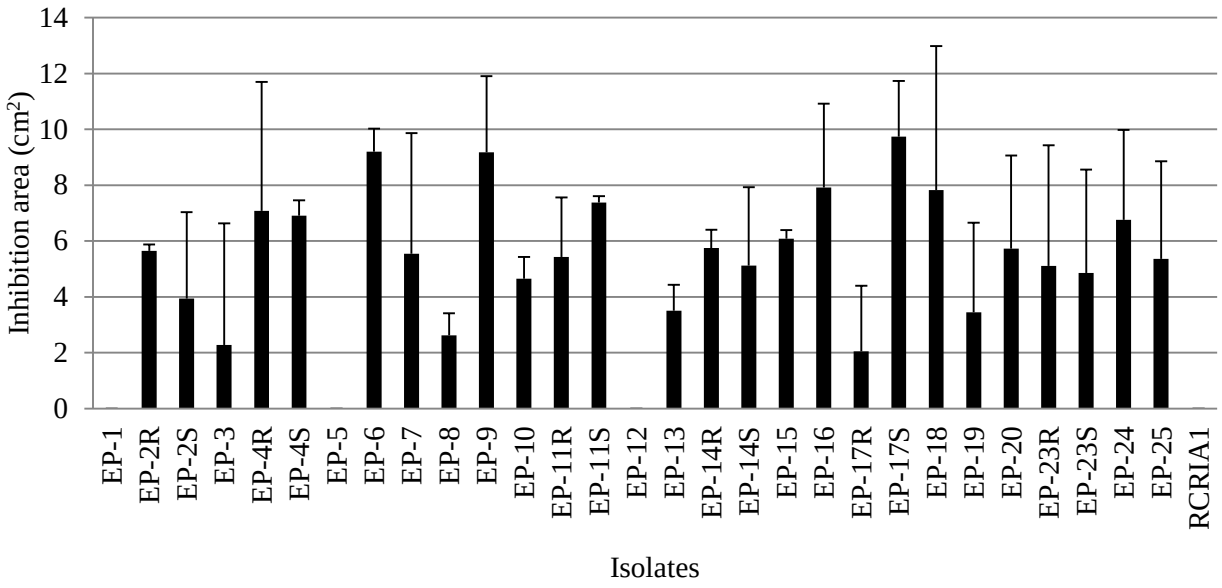

Figure 3.8 Antifungal activities of the rice leaf endophytes against *R. solani*. EP-1, EP5, EP-12 and RCRIA1 do not show antibacterial activity. EP-3, EP-8 and EP-17R have the lowest activity and EP-17S has the highest inhibition area with 9.7 cm^2. Each error bar indicates standard error from three replicates.

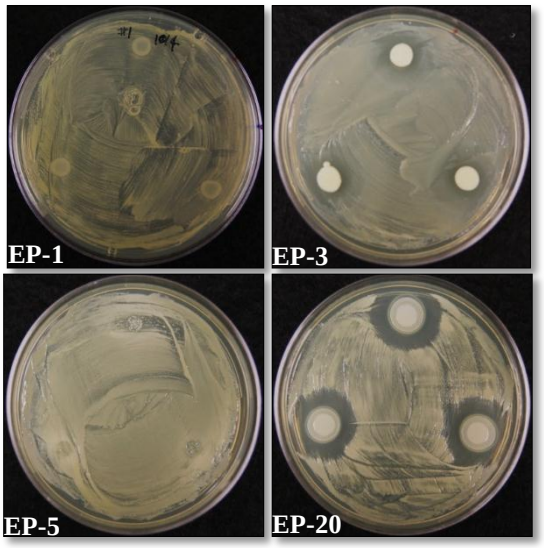

Figure 3.9 Antibacterial activities of the rice leaf endophytes on the PDA plates of some of the endophytes. Three spots in each plate are the spots of endophytes and *B. glumae* was spreaded on the media. EP-1 and EP-5 do not show any antibacterial activity, EP-3 has low activity in comparison to EP-20 against *B. glumae*.

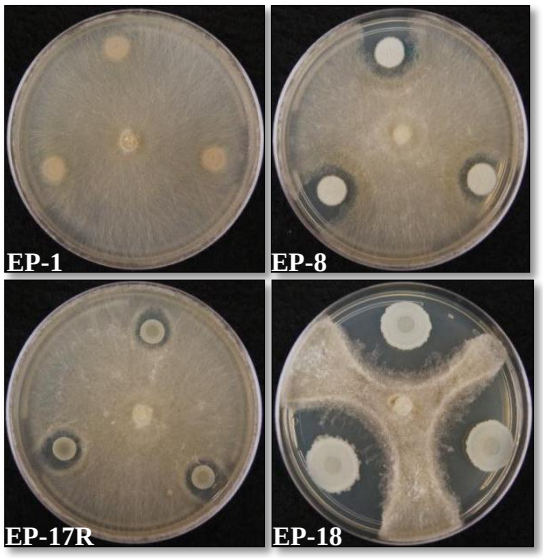

Figure 3.10 Antifungal activities of the rice leaf endophytes on the PDA plates of some of the endophytes. Three spots in each plate are the spots of endophytes and one plug of about 5 mm of *R. solani* was kept at the center of the PDA plate. EP-1 does not show any antibacterial activity, EP-8 and EP-17R have lower activity in comparison to EP-18 against *R. solani*.

4. DISCUSSION

Rice is the most important staple food around the world. Most people in south Asia and south East Asia rely on rice for the fulfillment of their major part of calories. However, various diseases and insect pests limit the production of rice that lead to the reduction of the rice yield. BPB is one of the emerging diseases of rice in southern USA, which causes about 60% yield loss in severely infested fields (Shahjhan et al., 2000). Panicle discoloration with unfilled grains is the characteristic symptoms of BPB. *B. glumae* is the major causal agent of BPB, which is favored by high night temperature with high relative humidity (Kurita et al., 1964). So, incidence of BPB may increases due to the ever global warming around the world. Toxoflavin, lipase, flagella, and type III secretion system are the major virulence factors of the *B. glumae* that causes diseases in rice and other field crops such as sesame, pepper, and eggplant (Jeong et al., 2003).

This bacteria was first reported in Japan causing grain rot and seedling rot (Goto and Ohata, 1956), and caused epidemic in 1990, 1995, 1998 and 2000 (Nandakumar et al., 2009; Shahjhan et al., 2000) in the rice growing southern states of the U. S. including Louisiana, Texas and Arkansas. Complete resistant cultivar for this disease has not been identified, however, partial resistant cultivar, Jupiter, has been reported to show less symptom development with higher yield (Sha et al., 2006). Previously conducted microarray analysis of gene expression in Jupiter found that several genes encoding grain filling proteins and transcription factors, were upregulated when challenged with *B. glumae* (Nandakumar and Rush, 2008).

The partial resistant cultivar, Jupiter, showed lesser symptoms than Trennase when inoculated with *B. glumae* 336gr-1. In addition, the toxoflavin deficient mutant of *B. glumae* also developed symptoms, but less than the wild type *B. glumae* 336gr-1, in both Jupiter and Trenasse (Fig. 2.3). Similar result was reported previously by Suzuki et al. (2004). This result suggests

that toxoflavin is not the only virulence factor of *B. glumae* to cause disease in rice, but there may be other virulence factors that cause BPB. Lipase is another virulence factor in *B. glumae*, which causes disease in rice and is quorum sensing dependent (Devescovi et al., 2007). Furthermore, a derivative of *B. glumae* which is impaired in both toxoflavin production and type III secretion system also induced symptoms in rice.

Induction of an NAC-like transcription factor (NTF) in Jupiter after the infection of the pathogen suggests that the NTF might be involved in expressing partial resistance in Jupiter. NAC transcription factors are involved in the growth and development of plants and in stress tolerance. However, sometimes they are also involved in inducing disease resistance in some plants as in potato where expression of *StNAC* induced after *Phytophthora infestans* infection and showed resistance (Collinge and Boller, 2001).

Interestingly, Os01g0393100 encoding the *NTF* is differentially expressed in Jupiter when inoculated with *B. glumae* 336gr-1 and *tox*$^{-}$ and *tox*$^{-}$*hrp*$^{-}$ derivatives (Fig 2.3). Os01g0393100 was highly induced in the panicles inoculated with toxoflavin deficient mutants of *B. glumae* in comparison to the *B. glumae* itself, and another mutant that is deficient in both toxoflavin production and HR, which suggests that toxoflavin may involve in the suppression of the Os01g0393100 in Jupiter. Furthermore, Os01g0393100 is induced more in the panicles inoculated with *B. glumae tox*$^{-}$*hrp*$^{-}$ than in the panicles inoculated with *B. glumae* wild type. However, it is less induced than in the panicles treated with toxoflavin deficient mutants. These results suggest that type III secretion system, encoded by the *hrp/hrc* genes may also be involved in the expression of the NTF encoding gene. Several NAC proteins involve positively and negatively in enhancing disease resistance against various bacterial, fungal as well as viral diseases. ATAF1 NAC proteins in *Arabidopsis* negatively regulates the disease resistance and

expression of defense related genes whereas HvNAC6, StNAC, OsNAC6 positively regulate the disease resistance in barley, potato and rice against *B. graminis* f. sp. *hordei*, *Phytophthora infestans* and *M. grisea* respectively (Collinge and Boller, 2001; Jensen et al., 2007; Nakashima et al., 2007).

Quantification of the induction of Os01g0393100 (*NTF* gene) in Jupiter showed about 70, 13 and 2 fold changes in the induction in panicles inoculated with *B. glumae tox*$^{-}$, *B. glumae tox*$^{-}$ *hrp*$^{-}$ and *B. glumae* 336gr-1, respectively, (Fig. 2.5). Even though the induction of Os01g0393100 (*NTF* gene) in Jupiter and Trenasse was different; its sequences along with the about 700 bp upstream region between both rice cultivars were identical. Analysis of the sequence with NCBI Basic Local Alignment Search Tool (BLAST) shows 100% identical with the sequence in NCBI rice genome database.

Results of our study showed that a grain filling protein, Os12g0269200, is also highly induced in Jupiter after the inoculation of *B. glumae* and its mutant derivatives. However, there is absence of induction in Trenasse even after the inoculation of bacteria, except in the panicles inoculated with *B. glumae tox*$^{-}$*hrp*$^{-}$. Os12g0269200 was up regulated in Jupiter, in a previous microarray experiment, after challenged with the pathogen (Nandakumar and Rush, 2008). This induction of prolamin in Jupiter suggests that it might play a crucial role in expressing partial resistance. Toxoflavin and type III secretion system in *B. glumae* may also involve in suppressing the expression of the grain filling protein, Os12g0269200, in Jupiter (Fig. 2.4).

These results together with the previously done microarray data suggest that the induction of Os01g0393100 and Os12g0269200 genes may be involved in the partial resistance of Jupiter. These genes can be used as a tool to develop BPB resistant rice varieties.

In an attempt to study the alternative methods of controlling BPB, several chemical compounds were tested for their disease suppression effects by pretreatment. In our study in 2010 summer, pretreatment of elicitor showed induction of disease resistance in rice reducing the disease symptoms, which is similar to the disease resistance and gene expression when challenged with pathogens via SAR (Gorlach et al., 1996). Ascorbic acid with its all three different concentrations significantly reduced the BPB symptoms in susceptible Trenasse among the pretreated elicitors (Fig. 3.1). Moreover, 200 µM of INA also reduced the disease severity in Trenasse. It has been reported earlier that INA restores disease resistance in tobacco and *Arabidopsis* (Delaney et al., 1995). CF with 100X dilution also reduced the BPB in rice (Fig.3.1); however, the mechanism of CF in reducing the BPB symptoms is still unknown. In addition to that, other pretreated elicitors, ET, JA and SA resulted in the lower disease severity score than the water pretreated control, but were not significant as compare to the one pretreated with ascorbic acid (Fig. 3.1).

Measurement of yield from those pretreated rice panicles indicated that ascorbic acid with 100 µM reduced the yield minimally (Fig. 3.2), whereas H_2O_2 along with other elicitors, ET, JA and SA pretreated rice panicles caused similar or lower yield than water pretreated panicles. 100X dilution of CF on the other hand produced higher yield (Fig. 3.2), but its mechanism remains unknown.

Repeated experiments in the greenhouse during the summer of 2011 showed a similar pattern of result in reducing the BPB symptoms. Pretreatment of panicles with AA again reduced the disease with all three concentrations; lowest with the 200 µM. In contrast, 10X dilution of CF got the highest disease severity score (Fig. 3.3). Yield data (Fig. 3.4) showed minimal reduction of yield in ascorbic acid pretreated rice. 100X dilution of CF have minimal reduction in yield as

compare to the non-inoculated panicles. Since ET did not show any prominent result in reduction of disease symptom and yield, it was not included in the greenhouse experiment.

However, 1st field experiment in 2011 summer did not show the significant reduction in disease symptoms, or effect in the yield in one of the susceptible cultivars, Bengal, by ascorbic acid pretreatment (Fig. 3.5 and Fig. 3.6). All of the panicles pretreated with different elicitors showed similar BPB symptoms and similar yield. Similarly, second planting of field experiments in 2011 summer, did not show significant results in the susceptible cultivar CL151 (Fig. 3.7 and Fig. 3.8). These results may be due to the adverse environmental situation prevailed in the summer of 2011. High speed storm and higher precipitation washed out the pretreated elicitors from the panicles, so proper activity of the elicitors could not be seen.

These studies showed that various elicitors and chemicals such as INA and ascorbic acid can be used to suppress the BPB in rice by enhancing the rice defense system and minimizing the effect of toxoflavin to rice plants, respectively, with minimal yield reduction. Ascorbic acid which showed significant reduction in disease symptoms with minimal yield reduction can be a good candidate to use to pretreat the rice plants for suppressing the BPB symptoms and for increasing rice yield. However, pretreatment of elicitors should be protected from rainfall and wind in the field conditions.

Similarly, activities of several endophytes isolated from different rice cultivars showed antagonistic effects against *B. glumae* 336gr-1 and *R. solani* (LR-71). In both antibacterial and antifungal activities, EP-1, EP5, and EP-12 did not show activity (Fig. 3.7 and 3.8). The endophytes showing strong antibacterial and antifungal activities could be good candidates to control BPB and sheath blight symptoms caused by *B. glumae* and *R. solani*. However, these are

only the biological assays done *in vitro*; effects of these endophytes on suppressing diseases need to be studied in the greenhouse as well as field conditions.

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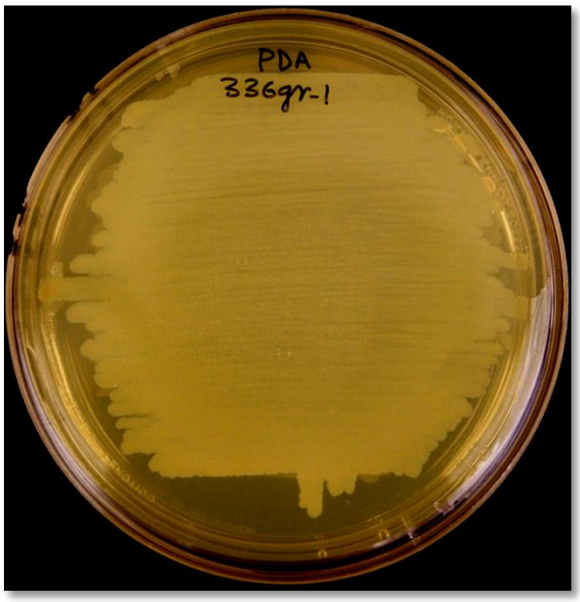
APPENDIX A: A TYPICAL BACTERIAL PANICLE BLIGHT SYMPTOMS IN RICE

Asymptomatic panicles

Symptomatic panicles

APPENDIX B: TOXOFLAVIN PRODUCTION IN POTATO DEXTROSE AGAR (PDA) BY THE *BURKHOLDERIA GLUMAE*



Toxoflavin production by virulent strain

No toxoflavin production by toxoflavin deficient mutant strain

APPENDIX C: NUCLEOTIDE SEQUENCE OF OPEN READING FRAME (ORF) OF GENE ENCODING NAC-LIKE TRANSCRIPTION FACTOR (NTF) OF JUPITER AND TRENASSE

Jupiter- ORF of NTF

ATGGGAGAGCAGCAACAGCAGGTGGAGCGGCAGCCGGACCTGCCTCCGGGCTTTAGGTTTCACC
CAACGGACGAGGAGATTATCACCTTTTACCTTGCACCCAAGGTTGTGGACAGCAGGGGCTTTTG
CGTTGCTGCCATTGGAGAGGTGGATCTCAACAAGTGCGAGCCATGGGATTTGCCAGGTAAACAT
TCTAGTATTATTTTGAATTGAGAGAAATTATATATATAATTAGGGAAATTTATATGATATGATA
TGATGCATGTGAAAAGTAAAAATAAATAAATGTAGGGAAGGCGAAGATGAATGGGGAGAAGGAG
TGGTATTTCTACTGCCAGAAGGATCGGAAGTACCCGACGGGGATGAGGACGAACAGGGCGACGG
AGGCTGGATACTGGAAGGCGACGGGGAAGGACAAGGAGATCTTCCGCGACCACCACATGCTCAT
CGGCATGAAGAAGACGCTCGTCTTCTACAAGGGCAGGGCTCCCAAGGGCGACAAGACCAACTGG
GTCATGCACGAGTACAGGCTCGCCGACGCCTCTCCGCCGCCGCCGCCATCCTCCGCAGAGCCCC
CGAGGCAGGACGACTGGGCCGTCTGCAGGATCTTCCACAAGAGCTCCGGCATCAAGAAGCCGGT
GCCGGTTGCTCCTCATCAGGTGCCCGCCGCCGCCAACTACCAGCAGCAGCAGCAGATGGCCATG
GCCTCCGCCGGCATCATCCAAGTCCCCATGCAGATGCAGATGCCATCCATGTCTGACCAGCTGC
AGATGTTGGACGACTTCTCCACCACCGCTTCACTCTCACTCATGGCGCCGCCTTCCTACTCCAC
TCTGCCTGCAGGCTTCCCGCTTCAGATCAACAGCGGCGCCCATCCCCAGCAGTTTGTTGGGAAC
CCGTCCATGTACTACCACCAGCAGCAGCAGATGGACATGGCCGGCGGAGGGTTCGTGGTGAGCG
AGCCGTCGTCGCTGGTGGTGTCGCCGCAGGATGCTGCCGACCAGAACAACAACGCCGCCGACAT
CTCGTCGATGGCATGCAACATGGACGCTGCCATCTGGAAGTACTGA

Trenasse- ORF of NTF

ATGGGAGAGCAGCAACAGCAGGTGGAGCGGCAGCCGGACCTGCCTCCGGGCTTTAGGTTTCACC
CAACGGACGAGGAGATTATCACCTTTTACCTTGCACCCAAGGTTGTGGACAGCAGGGGCTTTTG
CGTTGCTGCCATTGGAGAGGTGGATCTCAACAAGTGCGAGCCATGGGATTTGCCAGGTAAACAT
TCTAGTATTATTTTGAATTGAGAGAAATTATATATATAATTAGGGAAATTTATATGATATGATA
TGATGCATGTGAAAAGTAAAAATAAATAAATGTAGGGAAGGCGAAgATGAATGGGGAGAAGGAG
TGGTATTTCTACTGCCAGAAGGATCGGAAGTACCCGACGGGGATGAGGACGAACAGGGCGACGG
AGGCTGGATACTGGAAGGCGACGGGGAAGGACAAGGAGATCTTCCGCGACCACCACATGCTCAT
CGGCATGAAGAAGACGCTCGTCTTCTACAAGGGCAGGGCTCCCAAGGGCGACAAGACCAACTGG
GTCATGCACGAGTACAGGCTCGCCGACGCCTCTCCGCCGCCGCCGCCaTCcTCCGCAGAGCCCC
CGAGGCAGGACGACTGGGCCGTCTGCAGGATCTTCCACAAGAGCTCCGGCATCAAGAAGCCGGT
GCCGGTTGCTCCTCATCAGGTGCCCGCCGCCGCCAACTACCAGCAGCAGCAGCAGATGGCCATG
GCCTCCGCCGGCATCATCCAAGTCCCCATGCAGATGCAGATGCCATCCATGTCTGACCAGCTGC
AGATGTTGGACGACTTCTCCACCACCGCTTCACTCTCACTCATGGCGCCGCCTTCCTACTCCAC
TCTGCCTGCAGGCTTCCCGCTTCAGATCAACAGCGGCGCCCATCCCCAGCAGTTTGTTGGGAAC
CCGTCCATGTACTACCACCAGCAGCAGCAGATGGACATGGCCGGCGGAGGGTTCGTGGTGAGCG
AGCCGTCGTCGCTGGTGGTGTCGCCGCAGGATGCTGCCGACCAGAACAACAACGCCGCCGACAT
CTCGTCGATGGCATGCAACATGGACGCTGCCATCTGGAAGTACTGA

APPENDIX D: ALIGNMENT OF NTF SEQUENCE OF JUPITER AND TRENASSE USING CLUSTALW2

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Jupiter-NTF       ATGGGAGAGCAGCAACAGCAGGTGGAGCGGCAGCCGGACCTGCCTCCGGGCTTTAGGTTT 60
Trenasse-NTF      ATGGGAGAGCAGCAACAGCAGGTGGAGCGGCAGCCGGACCTGCCTCCGGGCTTTAGGTTT 60
                  ************************************************************

Jupiter-NTF       CACCCAACGGACGAGGAGATTATCACCTTTTACCTTGCACCCAAGGTTGTGGACAGCAGG 120
Trenasse-NTF      CACCCAACGGACGAGGAGATTATCACCTTTTACCTTGCACCCAAGGTTGTGGACAGCAGG 120
                  ************************************************************

Jupiter-NTF       GGCTTTTGCGTTGCTGCCATTGGAGAGGTGGATCTCAACAAGTGCGAGCCATGGGATTTG 180
Trenasse-NTF      GGCTTTTGCGTTGCTGCCATTGGAGAGGTGGATCTCAACAAGTGCGAGCCATGGGATTTG 180
                  ************************************************************

Jupiter-NTF       CCAGGTAAACATTCTAGTATTATTTTGAATTGAGAGAAATTATATATATAATTAGGGAAA 240
Trenasse-NTF      CCAGGTAAACATTCTAGTATTATTTTGAATTGAGAGAAATTATATATATAATTAGGGAAA 240
                  ************************************************************

Jupiter-NTF       TTTATATGATATGATATGATGCATGTGAAAAGTAAAAATAAATAAATGTAGGGAAGGCGA 300
Trenasse-NTF      TTTATATGATATGATATGATGCATGTGAAAAGTAAAAATAAATAAATGTAGGGAAGGCGA 300
                  ************************************************************

Jupiter-NTF       AGATGAATGGGGAGAAGGAGTGGTATTTCTACTGCCAGAAGGATCGGAAGTACCCGACGG 360
Trenasse-NTF      AGATGAATGGGGAGAAGGAGTGGTATTTCTACTGCCAGAAGGATCGGAAGTACCCGACGG 360
                  ************************************************************

Jupiter-NTF       GGATGAGGACGAACAGGGCGACGGAGGCTGGATACTGGAAGGCGACGGGGAAGGACAAGG 420
Trenasse-NTF      GGATGAGGACGAACAGGGCGACGGAGGCTGGATACTGGAAGGCGACGGGGAAGGACAAGG 420
                  ************************************************************

Jupiter-NTF       AGATCTTCCGCGACCACCACATGCTCATCGGCATGAAGAAGACGCTCGTCTTCTACAAGG 480
Trenasse-NTF      AGATCTTCCGCGACCACCACATGCTCATCGGCATGAAGAAGACGCTCGTCTTCTACAAGG 480
                  ************************************************************

Jupiter-NTF       GCAGGGCTCCCAAGGGCGACAAGACCAACTGGGTCATGCACGAGTACAGGCTCGCCGACG 540
Trenasse-NTF      GCAGGGCTCCCAAGGGCGACAAGACCAACTGGGTCATGCACGAGTACAGGCTCGCCGACG 540
                  ************************************************************

Jupiter-NTF       CCTCTCCGCCGCCGCCGCCATCCTCCGCAGAGCCCCCGAGGCAGGACGACTGGGCCGTCT 600
Trenasse-NTF      CCTCTCCGCCGCCGCCGCCATCCTCCGCAGAGCCCCCGAGGCAGGACGACTGGGCCGTCT 600
                  ************************************************************

Jupiter-NTF       GCAGGATCTTCCACAAGAGCTCCGGCATCAAGAAGCCGGTGCCGGTTGCTCCTCATCAGG 660
Trenasse-NTF      GCAGGATCTTCCACAAGAGCTCCGGCATCAAGAAGCCGGTGCCGGTTGCTCCTCATCAGG 660
                  ************************************************************

Jupiter-NTF       TGCCCGCCGCCGCCAACTACCAGCAGCAGCAGCAGATGGCCATGGCCTCCGCCGGCATCA 720
Trenasse-NTF      TGCCCGCCGCCGCCAACTACCAGCAGCAGCAGCAGATGGCCATGGCCTCCGCCGGCATCA 720
                  ************************************************************

Jupiter-NTF       TCCAAGTCCCCATGCAGATGCAGATGCCATCCATGTCTGACCAGCTGCAGATGTTGGACG 780
Trenasse-NTF      TCCAAGTCCCCATGCAGATGCAGATGCCATCCATGTCTGACCAGCTGCAGATGTTGGACG 780
                  ************************************************************

Jupiter-NTF       ACTTCTCCACCACCGCTTCACTCTCACTCATGGCGCCGCCTTCCTACTCCACTCTGCCTG 840
Trenasse-NTF      ACTTCTCCACCACCGCTTCACTCTCACTCATGGCGCCGCCTTCCTACTCCACTCTGCCTG 840
                  ************************************************************

Jupiter-NTF       CAGGCTTCCCGCTTCAGATCAACAGCGGCGCCCATCCCCAGCAGTTTGTTGGGAACCCGT 900
Trenasse-NTF      CAGGCTTCCCGCTTCAGATCAACAGCGGCGCCCATCCCCAGCAGTTTGTTGGGAACCCGT 900
                  ************************************************************

Jupiter-NTF       CCATGTACTACCACCAGCAGCAGCAGATGGACATGGCCGGCGGAGGGTTCGTGGTGAGCG 960
Trenasse-NTF      CCATGTACTACCACCAGCAGCAGCAGATGGACATGGCCGGCGGAGGGTTCGTGGTGAGCG 960
                  ************************************************************

Jupiter-NTF       AGCCGTCGTCGCTGGTGGTGTCGCCGCAGGATGCTGCCGACCAGAACAACAACGCCGCCG 1020
Trenasse-NTF      AGCCGTCGTCGCTGGTGGTGTCGCCGCAGGATGCTGCCGACCAGAACAACAACGCCGCCG 1020
                  ************************************************************

Jupiter-NTF       ACATCTCGTCGATGGCATGCAACATGGACGCTGCCATCTGGAAGTACTGA 1070
Trenasse-NTF      ACATCTCGTCGATGGCATGCAACATGGACGCTGCCATCTGGAAGTACTGA 1070
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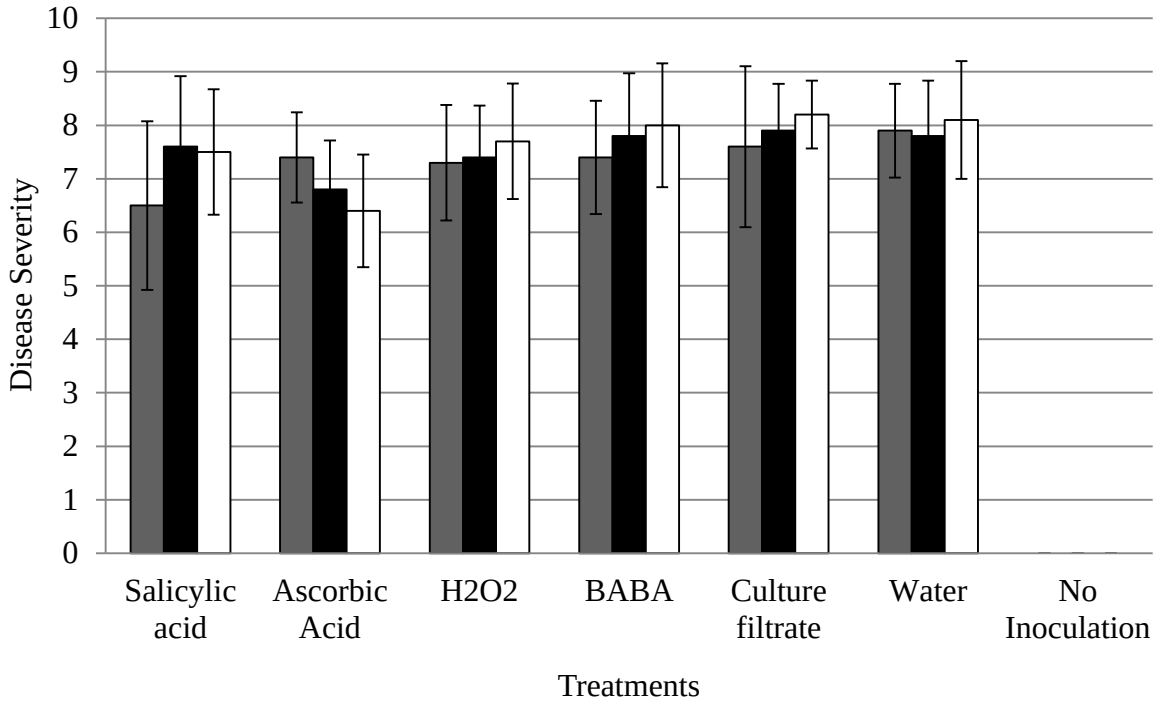
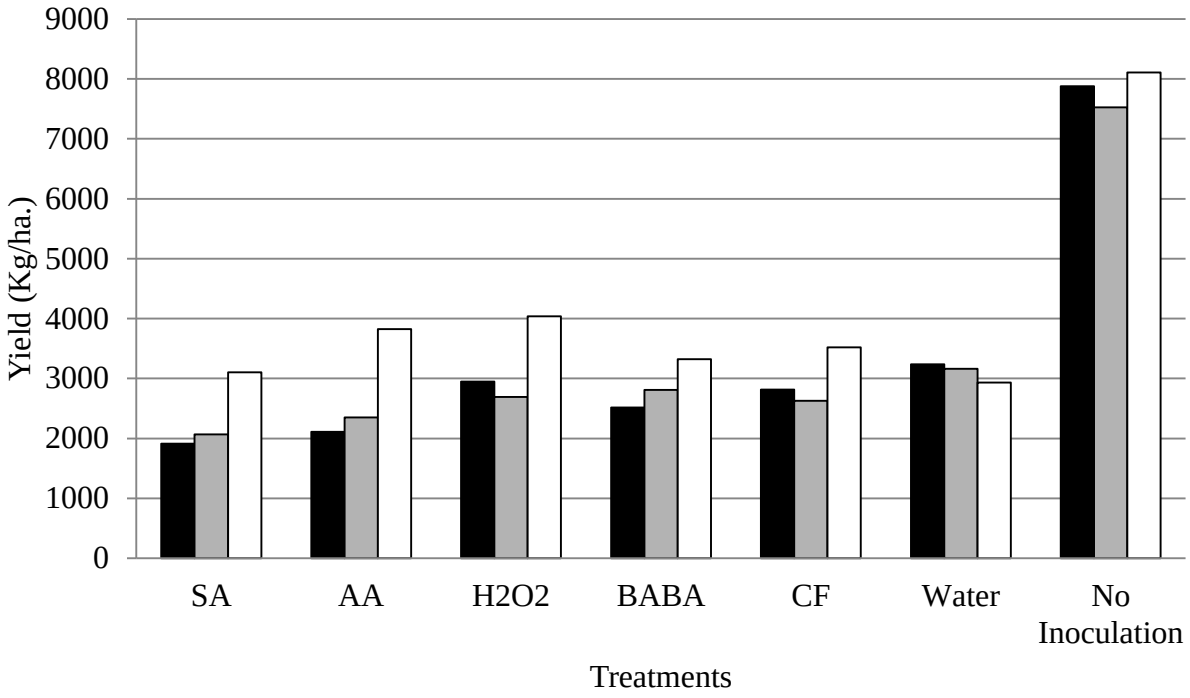
APPENDIX E: PRETREATMENT OF ELICITORS TO SUPRESS BPB IN RICE



Appendix E1. Ascorbic acid suppressed BPB symptoms in CL151. 2nd planting field data, 2011. Ascorbic acid suppressed BPB symptoms in rice. 1st planting field data, 2011. Disease rating on the panicles of Bengal was done 10 DAI of *B. glumae* 336gr-1. 1×10^8 CFU/ml of bacterial inoculum was inoculated 24 hours after the pretreatment of various elicitors each of with three different concentrations 100 µM, 200 µM, and 500 µM for SA; 50 µM, 100 µM, and 200 µM for AA and 0.5 µM, 5 µM, and 50 µM for H_2O_2; 50 µM, 100 µM, and 200 µM for BABA. However, culture filtrate was diluted only 100 times, and sprayed in three plots. Salicylic acid (SA), ascorbic acid (AA), hydrogen peroxide (H_2O_2), BABA, and culture filtrate (CF) were the elicitors used for pretreatment. Pretreatment with water was used as a positive control. No inoculation was used as a negative control. Disease scoring was done using standard scale (0-9) after 10 days. Each error bar indicates standard error from three replicates.



Appendix E2. CL151 yield (Kg/ha.) obtained from pretreated rice panicles (in 2nd field planting, 2011) by various elicitors each of with three different concentrations. Rice yield was weighed at 12% moisture condition. AA with 200 µM showed higher yield, but it is not significant as compare to other pretreated rows. Non inoculated row has the highest yield. Each error bar indicates standard error from three replicates.

APPENDIX F: MEASUREMENTS OF ANTIBACTERIAL ACTIVITIES

	Area of Inhibition zone Replication 1 (cm^2)	Area of Inhibition zone Replication 2 in (cm2)	Area of Inhibition zone Replication 3 in (cm2)	Mean	Standard Deviation
EP-1	0	0	0	0	0
EP-2R	5.053819015	4.561581625	4.043055212	4.55282	0.505438878
EP-2S	5.222897787	5.173155903	5.065163655	5.15374	0.080639772
EP-3	1.169370599	1.6534813	1.204277184	1.34238	0.269989499
EP-4R	5.123414019	4.856378644	4.867505118	4.9491	0.151063483
EP-4S	4.673991737	5.053764474	5.062545662	4.9301	0.221840255
EP-5	0	0	0	0	0
EP-6	4.673991737	4.528420369	4.717624968	4.64001	0.099073426
EP-7	3.914991678	3.957315913	4.552036855	4.14145	0.356209418
EP-8	3.473423378	5.012803778	3.672827245	4.05302	0.837156997
EP-9	5.243623571	3.096868592	5.772676501	4.70439	1.417062802
EP-10	4.867505118	-	4.45866174	4.45866	0.289095925
EP-11R	4.450589593	2.915790682	4.571017311	3.97913	0.922847561
EP-11S	4.55858184	2.419026343	4.021238597	3.66628	1.113067802
EP-12	0	0	0	0	0
EP-13	4.218406261	4.732242101	4.85376065	4.60147	0.337260836
EP-14R	4.55858184	4.604396733	5.344798127	4.83593	0.441291528
EP-14S	4.188790205	4.583234616	4.922482989	4.56484	0.367192264
EP-15	4.505349298	4.523893421	4.933554922	4.65427	0.24204906
EP-16	4.732242101	5.004513464	4.291982797	4.67625	0.359550623
EP-17R	4.703662334	5.028511741	4.283692483	4.67196	0.373420572
EP-17S	4.319689899	3.811799086	4.031710572	4.0544	0.25470448
EP-18	4.970915876	3.410155192	4.024074757	4.13505	0.786275953
EP-19	4.955207913	3.340560188	4.45866174	4.25148	0.827022416
EP-20	3.712533485	4.45582558	4.495968153	4.22144	0.441184852
EP-23R	4.671373743	4.867505118	4.819072231	4.78598	0.102166616
EP-23S	5.27263967	4.799655443	5.735151922	5.26915	0.467758008
EP-24	3.98698651	4.476769531	4.8038006	4.42252	0.411100552
EP-25	4.164573761	4.426809482	5.004513464	4.53197	0.42973018
RCRIA1	0	0	0	0	0

APPENDIX G: MEASUREMENTS OF ANTIFUNGAL ACTIVITIES

	Area of Inhibition zone Replication 1 (cm^2)	Area of Inhibition zone Replication 2 in (cm2)	Area of Inhibition zone Replication 3 in (cm2)	Mean	Standard Deviation
EP-1	0	0	0	0	0
EP-2R	5.445427266	5.895940379	5.594871083	5.64541	0.229469766
EP-2S	2.71835031	1.650863306	7.456046565	3.94175	3.089914966
EP-3	-1.22718463	0.904735051	7.157813429	2.27845	4.35802446
EP-4R	6.4289203	2.827433388	11.98932113	7.08189	4.615715047
EP-4S	6.509805402	7.295258107	-	6.90253	0.555398934
EP-5	0	0	0	0	0
EP-6	8.345291818	9.990919137	9.257226353	9.19781	0.824420905
EP-7	4.224569454	2.049234708	10.36725576	5.54702	4.313817923
EP-8	1.84306769	3.436771463	2.575015145	2.61828	0.797732489
EP-9	9.474519844	6.304783757	11.73624839	9.17185	2.728352706
EP-10	4.618141201	3.893175063	5.448263426	4.65319	0.778136516
EP-11R	5.015421772	3.541927551	7.738571737	5.43197	2.12910597
EP-11S	7.623761797	7.176139386	7.341073	7.38032	0.226377957
EP-12	0	0	0	0	0
EP-13	3.002838978	2.938261796	4.580398456	3.50717	0.930006893
EP-14R	6.207917983	5.280711818	-	5.74431	0.655633767
EP-14S	5.607961053	2.089813613	7.647814616	5.1152	2.811575451
EP-15	6.252423879	6.267477344	5.71769863	6.0792	0.313159793
EP-16	6.377433087	5.9725167	11.38041939	7.91012	3.012176419
EP-17R	0.79521564	0.586430629	4.764748858	2.0488	2.354397601
EP-17S	8.098327729	9.164723902	11.95812337	9.74039	1.993251271
EP-18	8.098327729	2.552544031	12.83427865	7.82838	5.146180054
EP-19	1.380719063	1.813615259	7.143850795	3.44606	3.209685793
EP-20	8.839001831	6.131559828	2.212859325	5.72781	3.331471655
EP-23R	1.687515221	3.671081915	9.9640847	5.10756	4.321227125
EP-23S	2.827433388	2.620611872	9.127417489	4.85849	3.698447741
EP-24	-	4.48026019	9.034042374	6.75715	3.220010263
EP-25	4.895648552	2.123847534	9.062622141	5.36071	3.492686235
RCRIA1	0	0	0	0	0

APPENDIX H: STATISTICALLY GROUPING OF THE SEVERAL TREATMENTS BASED ON THE DATA OBSERVED

Table H1: Kruskal-Wallis analysis for grouping of several chemicals/materials based on the disease score on Trenasse, Bengal and CL151 after the pretreatment in rice plants in the greenhouse, 2011, and the field, 2010 and 2011 at alpha=0.05.

2010 Field, Trenasse		2011 Greenhouse, Trenasse		2011 Field, Bengal		2011 Field, CL151	
Elicitors	**Statistical grouping**	**Elicitors**	**Statistical grouping**	**Elicitors**	**Statistical grouping**	**Elicitors**	**Statistical grouping**
HP	A	BGLM	A	BGLM	A	BGLM	A
Control	BI	Control	B	Control	B	Control	B
AA	BG	AA	C	JA	A	AA	C
CF	DEFGHI	INA	A	AA	A	SA	AC
INA	CDE	HP	A	BABA	A	HP	AC
SA	ACF	JA	A	SA	A	BABA	AC
JA	AE	SA	A	CA	A		
ET	AD	CF	A	HP	A		
BGLM	ACH			CF	A		

Observations with same alphabet are not significantly different at α=0.05.

Table H2: Tukey-Kramer analysis by Saxton macro of yield and grouping of several pretreated chemicals/materialson Bengal in the field, 2011 at alpha=0.05 (Saxton, 1998).

Obs.	Elicitor	Estimate	Standard Error	Letter Group
1	Control	436.33	44.8733	A
2	INA	387.09	25.9076	A
3	HP	361.80	25.9076	A
4	CA	358.67	25.9076	A
5	JA	351.49	25.9076	A
6	BABA	345.14	25.9076	A
7	AA	331.93	25.9076	A
8	1CF	318.02	22.4367	A
9	SA	309.97	25.9076	A
10	BGLM	268.56	44.8733	A

Observations with same alphabet are not significantly different at α=0.05.

VITA

The author was born in Chitwan, 80 mile south west to the capital city Kathmandu of Nepal as the eldest son of Mr. Ratna Lal Shrestha and Mrs. Prem Kumari Shrestha. He graduated his high school in 2000 near his hometown and received a bachelor's degree in agriculture science, major in plant breeding from Institute of Agriculture and Animal Sciences (IAAS), Tribhuvan University, Nepal in 2005. He worked as a research associate in REGARD-Nepal. He joined the Dr. Jong Hyun Ham's phytobacteriology lab in the Department of Plant Pathology and Crop Physiology at Louisiana State University in summer 2008 for further studies.