5.1. Introduction

Rice brown spot, caused by Bipolaris oryzae (Breda de Haan) Shoemaker (teleomorph: Cochliobolus miyabeanus (Ito and Kuribayashi) Drechs. ex Dastur.), is an orphan disease of rice, despite the fact that the disease chronically affects millions of hectares worldwide every year (Chakrabarti, 2001; Savary et al., 2000a; Zanao Junior et al., 2009). Reported yield losses in relative terms vary widely from 6 to 90%. Brown spot is by far one of the strongest yield reducers amongst rice diseases today (Barnwal et al., 2013). Infection of B. oryzae results in early senescence of the diseased plants, reduction of number of tillers, reduction of root and shoots elongation (Vidyasekharan et al., 1973).

Treatment of plants with various agents, including cell wall fragments, plant extracts and synthetic chemicals, can induce resistance to subsequent pathogen attack both locally and systemically (Walters and Fountaine, 2009). Such induced resistance rarely leads to complete pathogen control, resulting instead to a reduction in lesion size and/or number (Kuc, 1982). In broad terms, induced resistance can be split into two main types: systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR can be induced by treatment with a variety of agents, including necrotizing pathogens and certain chemicals (Spoel and Dong, 2012). ISR develops as a result of colonization of plant roots by certain strains of plant growth-promoting rhizobacteria (PGPR) and is mediated by a jasmonate (JA)- and ethylene (ET)-sensitive pathway (Spoel and Dong, 2012).

Induction of resistance can lead to the direct activation of defences, but can also lead to the priming of cells, resulting in stronger elicitation of those defences, or other defences, following pathogen attack (Goellner and Conrath, 2008). It seems likely that most induced resistance phenomena are based on a combination of direct induction and priming (Ahmad et al., 2010). Plants respond to the entry of potential pathogens by activating a battery of defense mechanism in which thousands of defense genes are coordinately expressed (Zhang et al., 2009). The most commonly studied genes that act as first line of defense are mitogen activated protein kinases (MAPKs), peroxidase (POX) and phenylalanine ammonia lyase (PAL). The speed of activation and the expression levels of defense gene and enzymes vary in different
plant pathogen interactions. In this report, we examined the expression of these genes and activities of POX and PAL enzyme levels in rice during *B. oryzae* infection.

Mitogen-activated protein kinases (MAPKs) are ubiquitous and evolutionarily conserved enzymes connecting cell surface to intracellular regulatory targets activating various morphogenetic changes (Eliahu *et al*., 2007). During the past decade, it has been firmly established that MAPKs play a central role in pathogen defense in rice (Nakagami *et al*., 2005). In the past few years, the number of publications revealing a function of MAPK cascades in pathogen signaling increased greatly. A set of MAPKs was found to be activated on interaction with pathogens, explaining why they are by far the most well known plant MAPKs. Other MAPKs might also be involved in plant-pathogen signaling, but they have been only rarely observed so far, possibly solely because of a lack of appropriate tools such as specific antibodies to detect low protein amounts or kinase activities.

Peroxidase (POX) catalyses the oxido-reduction of various substrates using hydrogen peroxide. Many reports have suggested that POXs play role in resistance to pathogens such as lignification and suberization (ref), cross-linking of cell wall proteins, xylem wall thickening, generation of reactive oxygen species, hydrogen peroxide scavenging, phytoalexin synthesis, antifungal activity of POX itself and auxin metabolism. Peroxidases are ubiquitous plant enzymes that are encoded by a large number of related genes within a plant genome (Passardi *et al*., 2004; Passardi *et al*., 2007) and have been implicated in a myriad of plant developmental process and responses to biotic and abiotic stress (Almagro *et al*., 2009; Casio and Dunand, 2009). An essential function for peroxidases is to protect the cellular membranes against oxidative damage. Peroxidases are players in both reactive oxygen species (ROS) removal and ROS generation (Passardi *et al*., 2005). Peroxidases have been documented as ROS scavengers in plants stressed by pathogens.

Phenylalanine ammonia-lyase (PAL) is the first enzyme of phenylpropanoid pathway that catalyzes the conversion of L-phenylalanine to trans-cinnamic acid. It is the key enzyme in the synthesis of several defense-related secondary compounds like phenols and lignins (Hemm *et al*., 2004). The presence of phenolic compounds in plants adn their synthesis in response to infection is associated with disease resistance.
Real-time quantitative PCR (RT-qPCR) has become a popular approach adopted by scientists since it is highly sensitive, accurate and rapid; provides high-throughput results; and can quantify mRNA copy numbers. Real time quantitative PCR remains one of the most sensitive and quantitative tools for gene expression used today. Quantitative PCR (qPCR) is a powerful, highly sensitive technique that can be used to quantitate gene expression and determine gene copy number. This study is the first report on expression of known defense genes in B. oryzae rice leaves by real time quantitative PCR.
5.2. Materials and Methods

5.2.1. Plant Materials and Growth Conditions

Rice varieties IET 13901 and Jaya were selected for their resistance and susceptibility to brown spot (BS) with the resistance scale of I and V respectively (based on Chapter IV). The experiment was carried out with two replications in randomized completely block design (RCBD) at the farmer’s field (Gejjagahalli, Mysore) during August 2013.

5.2.2. Chemical treatments under greenhouse condition

Jasmonic acid (JA) and salicylic acid (SA) were purchased from Sigma. 8 mM SA was directly dissolved in water containing 0.02% (v/v) Tween 20, whereas 2 µM JA was first dissolved in a few drops of methanol. For chemical treatment of plants, intact seedlings (6.5-leaf stage) were sprayed until near runoff with a fine mist of either compound. Control plants were sprayed evenly with a 0.02% (v/v) Tween 20 solution only. Foliar spray treatment was followed for application of chemicals and challenge inoculation of pathogen. Two days post-application, chemical-treated plants were challenged with *B. oryzae* as described above. Rice leaves were used for RNA extraction at 0, 1, 2, 3, 4 and 5 days post inoculation (dpi).

5.2.3. Biotic/Biocontrol treatments under greenhouse condition

*Pseudomonas fluorescens* and *B. oryzae* elicitor were used as biocontrol agents. Foliar spray treatment was followed for application of biocontrol agents and challenge inoculation of pathogen.

5.2.3.1. Preparation of *Pseudomonas fluorescens*

The bacterial strain *P. fluorescens* was obtained from the culture collections of the Department of Biotechnology, Mysore. It was grown in King’s medium B broth under constant shaking at 150 rpm for 48 h at room temperature (25±2°C). The culture at its stationary phase of growth was centrifuged at 6000 rpm for 10 min and bacterial cells were re-suspended in 10mM phosphate buffer (pH 7.0). The concentration was adjusted to 9 X 10^8 cfu/ml. then 2% carboxy methyl cellosolve was mixed with the bacterial suspension as a sticking agent and used as bacterial
inoculum. Plants were foliar spray treated with *P. fluorescens* cell suspension. Three days post application, *P. fluorescens*-treated plants were challenged with *B. oryzae* as described above. Rice leaves were used for RNA extraction at 0, 1, 2, 3, 4 and 5 days post inoculation (dpi).

### 5.2.3.2. Preparation of crude oligosaccharide elicitor from *B. oryzae*

To extract and purify the elicitor from *B. oryzae*, mycelial mat was grown on PDA supplemented with chloramphenicol antibiotic. The cultures were maintained on a rotary shaker at 125 rpm at 25°C for 10 days. The mycelial mats were harvested on the tenth day and blot-dried and stored at -20°C until further use.

The harvested mycelium of *B. oryzae* (25g) was powdered and left in acetone in (1:2.5v) at room temperature overnight by following the method of Nita-lazer *et al.* (2004). The residual powder was subjected to alkaline treatment in 10 ml of 0.1 M sodium hydroxide to 60°C for 2 h. The supernatant was collected by centrifugation (15,000 g for 15 min at room temperature) and neutralized with acetic acid and was stored overnight at 4°C. Later the supernatant was centrifuged (15,000 g for 15 min at room temperature). The supernatant obtained is the crude solution containing oligosaccharide. The presence of oligosaccharide in this solution was confirmed by Molisch test. The amount of reducing sugar present was quantified by the method of phenol-sulphuric acid detection (Dubois *et al.*, 1956).

Plants were foliar spray treated with *B. oryzae* elicitor. Two days post-application, *B. oryzae* elicitor-treated plants were challenged with *B. oryzae* as described above. Rice leaves were used for RNA extraction at 0, 1, 2, 3, 4 and 5 hours post inoculation (hpi) and 0, 1, 2, 3, 4 and 5 days post inoculation (DPI).

### 5.2.4. Pathogen inoculation under greenhouse conditions

*Bipolaris oryzae* strains BoKar, was grown for sporulation on potato dextrose agar at 28 °C. Seven-day-old mycelium was flattened onto the medium using a sterile spoon and exposed to blue light (combination of Philips TLD 18W/08 and Philips TLD 18W/33) for 3 d to induce sporulation. Upon sporulation, conidia were harvested as described by Thuan *et al.* (2006) and resuspended in 0.5% gelatin to a final density of 1 x 10^7 conidia mL^-1_. For inoculation, 5-week-old seedlings (6.5-leaf stage) were
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misted with conidial suspension (1mL per plant) using a sprayer. Immediately following inoculation, plants were moved into a dew chamber (30 °C ± 4 °C, 92% or greater relative humidity) to facilitate fungal penetration and, 18 h later, transferred to greenhouse conditions (28 °C ± 4 °C, 16-h-light/8-h-dark regime) for disease development. Control plants were treated with sterile distilled water instead of pathogen inoculum.

5.2.5. Integrated treatment under field condition

_Pseudomonas fluorescens_ seed treatment followed by foliar JA supply was used under field trials to check its efficiency in disease management. _P. fluorescens_ cell suspension as prepared earlier was used to treat seed of Jaya. Conical flasks containing seeds soaked in bacterial cell suspension was incubated overnight at room temperature with constant shaking. The primed seeds were air dried under sterile condition under the laminar air flow and used for sowing in fields. Seed were allowed to grow for 20 days in seed bed to obtain seedlings. After 20 days, seedlings were transplanted to wet fields and allowed to grow for 30 days. 30 day old plants were used in foliar JA treatment. Two days after JA treatment, plants were challenge inoculated with _B. oryzae_ conidial suspension as mentioned earlier. Control plants with only seed treatment, with only JA supply and with individual treatment with water and _B. oryzae_ were used to compare the efficiency of each treatment.

5.2.6. Sample harvest

Rice leaves were harvested from each treatment and their respective controls at known intervals. Rice leaves were used for RNA extraction at 0, 1, 2, 3, 4 and 5 hours post inoculation (hpi) and 0, 1, 2, 3, 4 and 5 days post inoculation (DPI).

RNA extraction, First strand cDNA synthesis, real-time quantitative PCR (qRT-PCR) was carried out according to the protocol mentioned in section.
5.3. Results

5.3.1. Effect of foliar elicitor treatment on brown spot incidence under greenhouse condition

Among the four elicitors used in the present study, foliar spray with *P. fluorescens* reduced the disease incidence to 53% compared to control plants (86% disease incidence). SA also reduced the brown spot incidence to 65%. Fungal elicitor and JA were less effective with 71 and 73% disease incidence respectively. Altogether, *Pseudomonas*, SA, fungal elicitor and JA provided 38, 29, 18 and 15% protection to brown spot disease (Fig. 5.1). The effect of different elicitors on brown spot severity is shown in Fig. 5.2.

![Figure 5.1. Effect of different elicitor treatment on brown spot disease incidence and protection under greenhouse condition](image)

5.3.2. Effects of foliar SA application on systemic induction of defense genes in *Bipolaris oryzae* infected rice leaves

Untreated plants showed down regulation of MPK12 through the analysis period, but was induced by 1.89-fold on 5 hpi. Foliar SA treatment of rice plants induced strong expression of MPK12 on 2 hpi with 59.12-fold increase compared to control plants that showed basal expression level. SA-induced MPK12 gradually dropped after 2 hpi but remained at higher levels till the end of analysis period. SA induced MPK12 expression was 26.15, 15.62 and 10.12-fold on 3, 4 and 5 hpi. mRNA level expression of MPK7 was maximum by 33.65-fold at 3 hpi in untreated-infected plants. However, SA-treated plants significantly induced MPK7 expression
on 1 hpi by 48.95-fold and subsequently decrease with time, but were maintained at higher expression levels (5.99-fold). MPK5 gene expression remained downregulated throughout the analysis period in untreated plants. SA treatment to rice plants showed upregulation of MPK5 expression from 1 hpi to 4 hpi; however there was no significant increase in fold change. Expression of MPK5 in SA-treated plants was found to be high on 5 hpi with 15.32-fold increase compared to control plants and untreated plants.

SA application to plants resulted in upregulation of mRNA levels of POX and PAL. Fold change in POX and PAL gene expression was high on 4 dpi and 3 dpi with 36.5-fold and 19.43-fold increase respectively. SA-induced PAL expression was 2.51 and 3.56-fold on 1 and 2 dpi respectively. It was maximum on 3 dpi and dropped on 4 and 5 dpi by 8.12 and 4.03-fold respectively. SA induced POX expression was 2.64, 4.96 and 6.78-fold on 1, 2 and 3 dpi respectively. SA induced POX expression peaked drastically on 4 dpi by 36.5-fold and was maintained at higher expression level of 20.86-fold on 5 dpi (Fig. 5.3.).

5.3.3. Effects of foliar JA application on systemic induction of defense genes in *Bipolaris oryzae* infected rice leaves

All three MPKs were induced upon foliar JA supply. The average fold change in MPK7 and MPK12 expression occurred between 1 to 2 hpi (hours post inoculation) indicating their early role in rice defense mechanism, whereas delayed expression was observed with MPK5 indicating its role in later stages of defense against *B. oryzae*. The first line of defense was related to MPK12 expression on 1 hpi in JA-treated plants, with 10.66-fold. mRNA level expression of MPK12 in JA-treated plants was 3.69, 3.22, 3.21 and 3.09-fold on 2, 3, 4 and 5 hpi respectively. The mRNA level of MPK7 was at basal level at 1 hpi in control plants, and increased by 1.2 times at 5 hpi. The expression of MPK7 was significantly upregulated on 3 hpi by 33.65-fold in untreated-inoculated plants. Fold change in MPK7 expression in JA treated plants was 10.56, 2.89 and 2.71-fold on 3, 4 and 5 hpi respectively.

In contrast, MPK5 gene remained above basal level expression with 1.32, 2.09, 2.26 and 2.49 fold on 1, 2, 3 and 4 hpi respectively in JA treated plants. A gradual but insignificant increase was observed in its expression that reached
maximum by 10.66-fold on 5 hpi. The expression remained downregulated upon pathogen infection throughout the analysis period. Control plants showed gradual increase in MPK5 expression and were slightly high compared to untreated-inoculated plants in terms of fold change.

Early upon infection (1 dpi), mRNA level expression of POX was induced by 1.5-fold and reached maximum on 4 dpi by 7.62-fold in untreated-inoculated plants compared to control plants. However, foliar JA application two-days prior to B. oryzae infection induced POX expression on 1 dpi with 1.03-fold and peaked maximum by 6.95-fold on 4 dpi. This fold change in POX expression was less in JA treated plants compared to untreated plants with pathogen infection alone.

Though, PAL is known to be induced by SA application, an attempt was made in the present investigation to study the effect of JA in systemic induction of PAL. Interestingly, it was found that foliar application of JA induced PAL expression. Early upon B. oryzae infection in untreated plants, mRNA levels of PAL were downregulated by 3.33-fold on 1 dpi. JA supply to plants induced PAL expression on 1 dpi (1.29 times), that increased significantly by 22.97-fold on 2 dpi. Fold change gradually decreased and maintained at slightly high levels (2.54-fold) on 5 dpi compared to control plants and untreated-inoculated plants with 1.47-fold increase and 1.03 fold reduction respectively (Fig. 5.3).

5.3.4. Effects of foliar B. oryzae-elicitor application on systemic induction of defense genes in Bipolaris oryzae infected rice leaves

Crude extract isolated from B. oryzae was used as elicitor to test for its efficacy in systemic induction of defense genes in rice plants. Fold change in MPK7 and MPK12 expression was found between 2 hpi to 3 hpi in B. oryzae elicitor treated plants. Upon fungal elicitor treatment, MPK12 expression was significantly induced at 2 hpi with 9.65-fold increase. The expression gradually decreased thereafter, but was maintained at slightly higher expression levels compared to the untreated plants that showed downregulation of MPK12 till the end of analysis period. Though, control plants showed gradual increase in MPK12 expression in time course, they were significantly low compared to treated plants. Expression of MPK7 increased significantly at 3 hpi (32.16-fold) in treated plants that was slightly less than the
expression obtained by pathogen infection alone with 33.65-fold change at same time interval. Changes in MPK7 expression were relatively low on 4 hpi and 5 hpi in treated plants compared to high expression in untreated plants with 12.5 and 3.71-fold change. MPK5 expression was induced late in fungal elicitor treated plants with a significantly high fold change of 49.25 times at 5 hpi. The expression of this gene was strongly induced in treated plants compared to the downregulated expression observed in untreated plants throughout the analysis period. Control plants maintained basal expression levels of MPK5 with slight changes.

Upon fungal infection, mRNA levels of POX were slightly induced above the basal expression levels at 1 dpi. A 7.62-fold increase was observed on 4 dpi that was maintained at 5.22-fold on 5 dpi. However, fungal elicitor induced POX expression earlier on 3 dpi with 7.86-fold, that was almost similar to POX expression induced by pathogen alone on 4 dpi. Change in POX was 2.06 and 2.03-fold on 4 and 5 dpi in treated plats that was very less compared to untreated-infected plants. Surprisingly, supply of fungal elicitor to plants resulted in downregulation of PAL expression on 1 dpi; however the downregulation was comparitively less in treated plants compared to untreated-infected plants. Upon fungal elicitor supply PAL expression returned to basal expression level on 2 dpi, subsequently increased and peaked high on 5 dpi with 6.69-fold increase compared to control plants (Fig. 5.3).

5.3.5. Effect of *Pseudomonas fluorescens* foliar application on induction of defense genes in *Bipolaris oryzae* infected rice leaves

*Pseudomonas fluorescens* was a strong inducer of all three MPKs in the present study. MPK7 and MPK12 expression was induced earlier at 1 hpi in *Pseudomonas* treated plants with striking increase of 46.97 and 33.47-folds compared to their respective control plants and untreated plants. In contrast to the untreated plants, where MPK12 remained attenuated throughout the analysis period, the expression of MPK12 significantly peaked by 33.47-fold at 1 hpi. The expression in *P. fluorescens* treated plants was 6.97, 3.66, 3.32 and 3.22-fold at 2, 3, 4 and 5 hpi respectively. MPK7 expression was induced earlier at 1 hpi in treated plants and maintained subsequently. Although, MPK5 expression was induced in treated plants at 1 hpi, there was no significant change in its expression. However, a sharp increase
by 18.69 fold was observed on 5 hpi that was very high compared to control and untreated plants.

Foliar supply of *P. fluorescens* 2-day prior to *B. oryzae* infection resulted in systemic induction of mRNA levels of POX that was 2.34 and 5.73 times higher at 1 and 2 dpi, which significantly peaked to 22.88-fold at 3 dpi. The expression gradually declined but was maintained at higher expression levels (10.56-fold) on 5 dpi compared to control and untreated plants. The *P. fluorescens* treated plants showed higher PAL expression compared to untreated and control plants. The expression was sharp on 2 dpi with 16.22-fold increase in the mRNA levels, but gradually declined and maintained at slightly higher expression level on 5 dpi.

Figure. Induction pattern of POX, PAL, MPK5, MPK7 and MPK12 genes by jasmonic acid (JA), salicylic acid (SA), fungal elicitor (FCE), *Pseudomonas fluorescens* (Pf) followed by challenge inoculation of *B. oryzae*. Samples were taken from each treatment at 0, 1, 2, 3, 4 and 5 hours post-inoculation for MPKs analysis and at 0, 1, 2, 3, 4 and 5 days post-inoculation for POX and PAL analysis. Quantitative reverse transcription-polymerase chain reaction data were normalized using the rice actin gene and are shown relative to 0 h (Fig. 5.3).

### 5.3.6. Integrated management and brown spot protection under field conditions

With this background, an integrated management strategy using seed treatment with *P. fluorescens* and foliar application of JA was tested for its effect on brown spot disease under field condition. Seed treatment with *Pseudomonas* alone followed by *B. oryzae* infection showed no significant changes in gene expression compared to control plants and untreated plants. Target gene expression was maintained at basal expression level throughout the analysis period in seed treated plants. Surprisingly, seed treatment with *Pseudomonas* followed by *B. oryzae* infection provided 43% disease protection, indicating seed treatment is more effective than foliar spray treatment that provided 38% protection to brown spot disease. However, JA treatment to rice plants as foliar spray under field condition had higher protection (24% protection) provided under green house condition (15% protection) (Fig. 5.4). Brown spot severity is compared between untreated-inoculated control and *Pseudomonas* seed treated plants supplied with foliar JA and followed by pathogen
challenge (Fig. 5.5.). A combined supply of *P. fluorescens* as seed treatment followed by foliar spray treatment of JA resulted in significant gene expression and disease protection (56%) against brown spot disease under field conditions. Gene expression in combined treatment plants was induced earlier than the gene expression observed in the individual treatment under greenhouse conditions.

![Figure 5.2. Effect of different elicitor treatment on brown spot severity. a-Uninoculated control, b- inoculated control, c- *Pseudomonas fluorescens* treatment, d- Salicylic acid treatment, e- Crude fungal elicitor, f- Jasmonic acid.](image)
Figure 5.3. Induction pattern of POX, PAL, MPK5, MPK7 and MPK12 genes by different treatments under greenhouse and field conditions. Relative gene expression of genes were compared with individual jasmonic acid (JA), salicylic acid (SA), Crude fungal elicitor (E) and *Pseudomonas fluorescens* (P) followed by challenge inoculation of *B. oryzae*. Under greenhouse conditions with gene expression in plants with seed treatment combined with foliar JA supply. Samples were taken from each treatment at 0, 1, 2, 3, 4 and 5 hours postinoculation for MPKs analysis and at 0, 1, 2, 3, 4 and 5 days postinoculation for POX and PAL analysis. Quantitative reverse transcription-polymerase chain reaction data were normalized using the rice actin gene and are shown relative to 0 h.
Integrated disease management and target gene expression under field conditions

Control plants with no treatment and no pathogen challenge showed gradual increase in the expression of all target genes but no significant changes were observed. Pathogen challenge to untreated plants under field conditions showed similar gene expression pattern with slight differences in fold change. POX expression in field experiments were almost similar to greenhouse experiments, but fold change in its expression was slightly lower in untreated rice plants challenged with pathogen under field conditions. POX expression was 5.66 fold and 7.62 fold under field and greenhouse conditions respectively. Exogenous JA treatment under field conditions resulted in higher expression on POX by 7.69 fold on 3 dpi compared to greenhouse treatment with 6.95 fold POX on 4 dpi. Seed treatment with *Pseudomonas* had no significant effect on POX expression under field conditions; however, foliar treatment with *Pseudomonas* under greenhouse conditions increased POX expression by 22.88 fold on 3 dpi. Seed treatment with *Pseudomonas* followed by JA treatment and pathogen challenge resulted in higher expression of POX with 38.65 fold on 3 dpi and subsequently maintained at higher expression levels of 29.81 and 27.22 fold on 4 and 5 dpi respectively.

PAL expression was almost similar in their respective controls under greenhouse and field conditions. It was observed that PAL was slightly high in control plants under greenhouse conditions. PAL expression was slightly higher in untreated plants challenged with *B. oryzae* under field conditions; however PAL expression remained downregulated (3.05 fold reduction on 1 dpi) in control plants under field conditions also downregulation in PAL was less compared to greenhouse condition (3.33 fold reduction on 1 dpi). Comparison study on the effect of foliar JA supply on PAL expression under greenhouse and field conditions varied significantly. PAL expression peaked maximum at same time interval in both field and *in-vitro* trials with 36.69 and 22.97 fold respectively. It was also observed that durability of PAL was stronger in field trials compared to *in-vitro* trials. Seed treatment with *Pseudomonas* had no significant effect on PAL expression under field trials; however, foliar treatment with *Pseudomonas* under greenhouse conditions increased PAL expression by 22.97 fold on 2 dpi. Seed treatment with *Pseudomonas* followed by JA treatment and pathogen challenge resulted in higher expression of PAL with 29.63
fold on 1 dpi and subsequently maintained at higher expression levels of 19.61, 8.42, 8.19 and 7.93 fold on 2, 3, 4 and 5 dpi respectively.

![Graph showing disease incidence and protection under greenhouse condition.](image)

**Figure 5.4.** Effect of different elicitor treatment on brown spot disease incidence and protection under greenhouse condition

![Image showing treatment effects under field conditions.](image)

**Figure 5.5.** Effect of *Pseudomonas* seed treatment and foliar JA treatment on brown spot disease incidence under field condition. a- Control plants (Jaya) challenged with *B. oryzae* with no pre-treatment; b- *Pseudomonas* seed treated plants and foliar JA supplied plants challenged with *B. oryzae*

Similar to *in-vitro* trials, MPK5 expression remained downregulated in untreated-challenged plants throughout the analysis period under field trials. The downregulation of MPK5 expression was slightly less under field trials compared to
in-vitro trials. Exogenous JA treatment had no significant effect on MPK5 expression under field trials, but the expression levels were slightly higher in terms of fold change observed under field trials with 3.92 fold on 5 hpi. Field trial with Pseudomonas seed treatment maintained MPK5 at basal expression level with no significant change but with gradual increase. In-vitro trials with foliar Pseudomonas treatment induced MPK5 expression by 18.69 fold on 5 hpi. Integrated treatment using Pseudomonas and JA followed by pathogen challenge slightly induced MPK5 expression by 1.96 fold on 1 hpi and peaked maximum by 29.23 fold on 5 hpi.

A significant increase of MPK7 by 61.16 fold on 1 hpi was observed upon combined seed treatment with Pseudomonas and foliar JA supply followed by pathogen challenge under field trials. Individual foliar supply of JA and Pseudomonas under in-vitro trials increased MPK7 expression by 16.28 and 46.97 fold Ron 2 hpi and 1 hpi respectively. Untreated control plants without pathogen inoculation and plants with Pseudomonas seed treatment followed by pathogen challenge had similar MPK7 expression with gradual increase but no significant changes under field condition.

Pathogen inoculation to untreated plants under in-vitro conditions resulted in downregulation fo MPK12 throughout the analysis period, whereas under field trials the downregulation was less from 1 to 4 hpi and was induced on 5 hpi by 1.75 fold. JA treatment followed by pathogen challenge induced higher expression levels of MPK12 by 19.41 fold and 10.66 fold on 2 hpi and 1 hpi under field and green house trials. Pseudomonas as seed treatment under field trials had negligible effect on MPK12 expression, whereas foliar Pseudomonas treatment under in-vitro trials increased MPK12 expression by 33.47 fold on 1 hpi. Seed treatment with Pseudomonas and foliar JA treatment followed B. oryzae infection severely induced MPK12 expression on 1 hpi by 69.73 fold and subsequently maintained at higher expression levels of 41.28, 22.61, 18.39 and 10.98 fold on 2, 3, 4 and 5 hpi respectively (Fig. 5.6).
**Figure 5.6.** Induction pattern of POX, PAL, MPK5, MPK7 and MPK12 genes by different treatments under greenhouse and field conditions. Relative gene expression of genes were compared with individual jasmonic acid (JA) and *Pseudomonas fluorescens* (P) followed by challenge inoculation of *B. oryzae*. Under greenhouse conditions with gene expression in plants with seed treatment combined with foliar JA supply. Samples were taken from each treatment at 0, 1, 2, 3, 4 and 5 hours postinoculation for MPKs analysis and at 0, 1, 2, 3, 4 and 5 days postinoculation for POX and PAL analysis. Quantitative reverse transcription-polymerase chain reaction data were normalized using the rice actin gene and are shown relative to 0 h.
5.8. Discussion

The expression of five genes encoding for PAL, POX, MPK5, MPK7 and MPK12 were examined in rice leaves upon *B. oryzae* infection. All genes were up-regulated in rice plants induced with different elicitors; the relative expression levels ranging from 1.03 to 59.12-fold under greenhouse condition. Differences in fold change were observed among the genes and among different elicitors used. In general, the highest mRNA levels were found in SA-treatment and *Pseudomonas* treatment. Gene expression was induced earlier in *Pseudomonas* treated plants compared to SA treated plants, but both of them showed durable resistance. On the other hand, JA and fungal elicitor treatment resulted in comparatively less gene expression with less durability in their role in brown spot resistance.

SA was a strong inducer of all three MPKs in the present investigation. The expression of MPK5, MPK7 and MPK12 was 15.32, 48.95 and 59.12 folds in SA treated plants compared to 3.66, 16.28 and 10.66 folds in JA-treated plants. Though, SA and JA treatment had differential effect on MPK5 expression its delayed expression upon treatment indicates its role in later stages of host-pathogen interaction. Bipolaris oryzae infection in untreated rice resulted in down regulation of MPK5 throughout the analysis period; this may be an indication that mRNA of MPK5 gene is degraded upon pathogen infection. In untreated-infected plants, the changes in the down regulation of MPK5 to reach the basal expression level during later stages of *B. oryzae* infection may be an indication that MPK5 plays its role in later stages of rice-*B. oryzae* pathosystem. However, SA or JA supply individually induced MPK5 expression during early stages of infection, a significant change was only observed at later stage of infection on 5 hpi. It was also observed that MPK5 expression was almost same with slight differences in fold change from 1 to 4 hpi in both SA and JA individual treatment. A remarkable change was observed only on 5 hpi in SA treatment with 15.32 fold of MPK5 expression that was very less compared to JA treatment with 3.66 fold at same time interval. This indicates SA as a strong inducer of MPK5.

MPK12 was another MPK gene that remained downregulated upon pathogen infection in untreated plants throughout the analysis period but reached basal
expression level on 5 hpi. A drastic increase in MPK12 expression by 59.12 fold on 2 hpi was induced upon SA treatment compared to a 10.66 fold change on 1 hpi in JA treated plants. SA induced MPK12 were consistently maintained at higher expression level compared to JA induced MPK12 indicating SA as its strong inducer. A significant upregulation by 33.65 fold of MPK7 was observed on 3 hpi in untreated plants. SA had a major role in MPK7 expression with a highest of 48.95 fold change on as early as 1 hpi. The expression decreased subsequently but was maintained at higher levels compared to control and untreated-infected plants. JA treatment also induced MPK7 expression, but was comparatively low to SA induced MPK7 expression. SA’s durability was more in all MPKs expression compared to JA.

There was no significant change in POX expression in JA-induced plants compared to SA-induced plants. It was also observed that SA-induced POX was maintained at higher levels (20.86 fold) on 5 dpi compared to JA-induced POX that was slightly high above basal level expression (2.38) on same time interval. Though, PAL is induced by both JA and SA (individual treatment), in the present study, JA is considered as a strong inducer of PAL compared to SA in terms of fold change. Surprisingly, it was also observed that SA was responsible for late (3 dpi) induction of PAL with less (19.43 fold) expression compared to JA-induced PAL that were induced earlier on 2 dpi with 22.97 fold increase. Though SA-induced PAL were late and less in terms of fold change compared to JA-induced PAL, mRNA levels of PAL were maintained at higher levels in case of SA treatment till 5 dpi, whereas high and early JA-induced PAL reached basal expression levels on the same time interval. POX and PAL activity changes upon MeJA, SA and MeSA were observed by Mandal, 2010.

Fungal elicitor treatment and Pseudomonas fluorescens treatment also induced the target gene expression during rice-B. oryzae interaction. Efficiency of Pseudomonas treatment in gene expression was high compared to fungal elicitor in terms of gene fold change. Among the three MPKs, MPK7 and MPK12 were induced earlier on 1 hpi with 46.97 and 33.47 fold change respectively, compared to fungal elicitor treatment that induced the expression of these two genes slightly above basal expression levels. Fungal elicitor treatment induced MPK7 and MPK12 expression on 3 hpi and 2 hpi by 32.16 and 9.65 fold. This indicates that Pseudomonas treatment played a major role in early and high induced gene expression compared to fungal
elicitor that was responsible for late and relatively low gene expression. The durability of gene expression was comparatively high in *Pseudomonas* treated plants than in fungal elicitor treated plants. Fungal elicitor was found to be a strong inducer of MPK5 with a significant of 49.25 fold change on 5 hpi. The expression of MPK5 was 18.69 in *Pseudomonas* treated plants at the same time interval. This indicates both elicitors (fungal elicitor and *Pseudomonas*) induced MPK5 expression at later stages of host-pathogen interaction, of which fungal elicitor is considered to be a strong inducer of MPK5.

POX and PAL were differentially expressed upon fungal elicitor and *Pseudomonas* treatment. *Pseudomonas* was a strong inducer of both POX and PAL with 22.88 and 16.22 fold changes on 3 and 2 dpi respectively. POX gene was strongly induced upon fungal elicitor on 3 dpi by 7.86 fold change, whereas PAL expression was delayed with 6.69 fold change observed on 5 dpi. The durability of *Pseudomonas* treatment was strong in terms of fold change throughout the analysis of target gene expression compared to fungal elicitor treatment.

Among all the four elicitors tested for target gene expression, SA and *Pseudomonas* were highly effective, where SA stood for high expression and durability of fold change in gene expression, whereas *Pseudomonas* treatment led to comparatively early induction of all the genes.

It has been also observed that foliar supply of SA and *Pseudomonas* as individual treatment followed JA-induced pathway by strongly upregulating the expression of MPK7, MPK12 and MPK5 genes. The activation of these genes in series may be involved in the activation of POX expression. Although, JA is a strong inducer of POX, its expression was strongly induced by SA supply in the present study. Fungal elicitor and JA supply to plants followed SA-signaling pathway resulting in upregulation of PAL and MPK7. The upregulation of MPK7 was induced strongly upon individual treatment with SA and JA, indicating this gene may be co-induced by both the elicitors. All these observations indicate SA-JA cross talk might have played its role in rice defense signaling pathway. The present work is a baseline to understand the role of these genes in rice-*B. oryzae* interaction. However, the exact role of the genes in the rice defense mechanism against *B. oryzae* has to be further proved by mutant analysis and transgenic studies.
Two interesting factors were observed with JA treatment under field conditions. First, foliar JA treatment alone provided 31% protection under field conditions. The protection was almost doubled compared to foliar JA supply under greenhouse conditions (15% protection). Second, gene expression was also high under field conditions compared to foliar JA treatment under greenhouse condition. JA treatment under greenhouse condition was provided to 15 day old seedlings whereas the same treatment was given to 50 day old rice plants. This includes rice as seedlings in seed bed (20 day seedlings) that are transplanted to wet fields and well maintained to get 30 day old rice plants. Thus, under field condition JA treatment will be provided to 50 day old seedlings whereas under greenhouse condition, very young 15 day old seedlings are treated with JA. This may be an indication that JA effect on rice plants is age-dependent and also depends on the environmental conditions. It may be believed this phenomenon is linked to ‘age-related resistance’ in rice (Develey-Riviere and Galiana, 2007); however the molecular mechanism of age related resistance is poorly understood. Reports suggest that the establishment of age-related resistance may require activation of SA dependent pathway in rice (Xie et al., 2011).

Seed treatment with *Pseudomonas* had no effect on gene expression was maintained at basal expression level with slight or negligible changes. This may be an indication that *Pseudomonas* had no systemic but localized protection against brown spot. In other words, *Pseudomonas fluorescens* in the present study lead to a type of systemic resistance, commonly denoted as induced systemic resistance (ISR) (Bakker et al., 2003; van Loon et al., 1998). Generally, the onset of ISR, unlike SAR, is not accomplished by the concomitant activation of PR genes (Van Wees et al., 1999). Instead, recent research revealed that ISR-expressing plants are primed to react faster to pathogen attack (Verhagen et al., 2004). This is in agreement with the observations made in the present study where foliar spray with *Pseudomonas* followed by *B. oryzae* infection increased gene expression, whereas seed treatment had no significant effect. Nevertheless, seed treatment was more effective than foliar spray treatment by providing 43% protection compared to 38% protection through foliar spray. This is supported by the fact that activation of SAR requires the translocation of a factor from the avirulent pathogen inoculated leaf to the distal leaves (Makandar et al., 2010). It was observed that avirulent strain of *Pseudomonas* syringae pv. tomato reduced the severity of disease in Arabidopsis caused by *Fusarium graminearum*. The SAR
activating factor is present in the petiole exudates (PeX) of Arabidospis. When this PeX was applied to lower leaves of Arabidopiss, it was found to protect upper leaves against F. graminearum indicating SAR is activated in Arabidopiss. Foliar spray treatment in the present investigation with \textit{P. fluorescens} activated SAR by decreasing the disease severity. Similarly, seed treatment also protected the rice plants against brown spot disease with highest protection level (43%) compared to all other treatments. SAR in rice has been previously described by Smith and Metraux (1991), where \textit{P. syringae pv. syringae} provided enhanced resistance to \textit{Magnaporthe grisea}. However, Reimmann and associates (1995) failed to reproduce these results, indicating that conditions for SAR are critical. In rice, colonization of the rhizosphere with the PGPR strains \textit{P. fluorescens} PF1 and PF7 enhanced resistance against sheath blight disease (Nandakumar \textit{et al.}, 2001). Someya and associates (2002, 2005) reported induced resistance to rice blast and sheath blight by the antagonistic bacterium \textit{Serratia macescens} B2. \textit{Pseudomonas} aeruginosa 7NSK2-mediated ISR effectively protected rice against \textit{M. grisea} (leaf blast fungi) but failed to consistently reduce sheath blight severity caused by \textit{Rhizoctonia solani}). This is due to spatial separation of inducing bacteria and challenging pathogens throughout the experiments, indicating \textit{Pseudomonas} aeruginosa 7NSK2-induced protection is plant mediated (De Vleesschauwer \textit{et al.}, 2006).

Seed treatment with \textit{Pseudomonas} had no effect on gene expression in the present study. This is in contrast with the previous view where increased disease protection was in correlation with the increased gene expression. The ‘silent factor’ in \textit{Pseudomonas} seed treated plants has to be investigated that had negligible effect on gene expression but provided maximum protection against brown spot disease. It may be predicted that this ‘silent factor’ present in the seeds as ‘priming inoculum’ could not be translocated to distal leaves to provide protection against the leaf spot pathogen-\textit{B. oryzae}. If this factor could not be translocated to the distal leaves to induce gene expression, how could it provide protection against brown spot disease? This puzzle has to be solved by further investigation to look whether single or multiple factors in combination are working together during rice-\textit{B. oryzae} interaction. Unraveling these factors may be greatly implied in transgenics and plant breeding.
Seed treatment with *Pseudomonas* followed by pathogen inoculation had no significant impact on target gene expression throughout the analysis period, however maintained at basal expression level. The gradual increase in expression of all genes in control plants in the present study indicates that gene expression increases with time and age. Gene expression was slightly higher in rice plants in field trials than the rice seedlings used in invitro trials, indicating that all these genes may be developmentally regulated in rice. Individual foliar treatment with JA under field conditions induced higher levels of all genes in the present study. Gene expression was induced earlier and durable with high expression levels compared to invitro JA treatment. In both the trials, PAL gene was expressed at higher levels. Field trial showed 36.69 fold increase whereas invitro trials showed 22.97 fold change in foliar JA treated plants. Based on this it may be concluded that exogenous supply of JA to rice induced SA-signaling pathway that is in agreement with high expression of PAL. Also, PAL was expressed at higher levels in older leaves (field trial) than in younger leaves (invitro trials), suggesting SA biosynthesis is regulated by leaf development. Similar results were described by Xie *et al.* (2011), where PAL gene, encoding key enzymes for SA synthesis in phenylpropanoid pathway was expressed in higher levels in old leaves than in new leaves. Apart from PAL, MPK7 expression was also highly upregulated in JA treatment under field trials, by 31.94 fold that was subsequently maintained at higher expression levels compared to invitro trials (16.28 fold). Based on the present investigation and related studies made by Xie *et al.* (2011), it may be hypothesized that SA-dependent pathway is developmentally regulated and more active in old leaves in rice.

Exogenous supply of JA alone induced POX expression in both field and invitro trials. The efficiency of JA to induce both PAL and POX in the present study indicates that JA plays a significant role in PR gene induction and resistance in rice (Mei *et al.*, 2006). The early induced POX upon JA treatment in field trials is in correlation with the findings that exogenous supply of JA induces rapid accumulation of PR proteins in old leaves but not in new leaves. However, the rapidity was less with no major difference in fold change observed in POX expression, whereas it was true in case of PAL expression. Moreover, MPK5 gene expression had no significant changes in fold change. This may be an indication that JA had least or no effect in MPK5 expression in either of the trials. Exogenous JA induced a significant and rapid
accumulation of MPK7 and MPK12 under field trials compared to *invitro* trials. Descriptions made in previous studies and based on the present investigation, it may be hypothesized that a relatively active JA signaling pathway together with the SA pathway potentiates a state of pathogen resistance in old leaves.

Interestingly, a combination of seed treatment with *Pseudomonas* and foliar spray treatment with JA to manage brown spot disease was highly effective providing 56% disease protection and dramatic and durable expression of all target genes in the present study. Highest expression of MPK12 was induced by 69.73 fold on 1 hpi, followed by MPK7 with 61.16 fold upregulation at same time interval that were subsequently maintained at highest expression levels. MPK5 expression was also high with 29.23 fold on 5 hpi. POX and PAL were induced on 3 dpi and 1 dpi by 38.65 and 29.63 fold increase.

However, the observations made in the present study have to be tested in large scale with different rice varieties under different environmental conditions for its durability in protection against brown spot disease caused by *B. oryzae*.