Chapter IV: Biochemical and Molecular Changes During Rice-B. oryzae Interactions

4.1. 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 shoot elongation (Vidyasekharan et al., 1973).

Host plant resistance to disease is an effective and economical way to manage brown spot. However, breeding efforts have emphasized acute diseases such as leaf blast and bacterial blight rather than chronic diseases such as brown spot (Savary et al., 2011) despite the importance of brown spot. The search for sources of resistance to brown spot has been a long-standing effort (Chakrabarti, 2001). There are very few reports on sources of resistance among rice varieties, however attempts have been made by Satija et al., 2005 and Shukla et al., 1995.

Pathogen infection is one of the major environmental stresses that affects growth and metabolism of plants. To survive under pathogen challenges, plants have developed delicate and complicated defense strategies through multiple signalling pathways (Peng et al., 2012). Direct assessment of structural, biochemical and physiological changes during disease development has identified some putative defense responses in rice disease resistance (Song and Goodman, 2001). On the other hand, genetic and molecular techniques have isolated several defense-related genes involved in rice disease resistance (Muttukrishnan et al., 2001).

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, 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 and their synthesis in response to infection is associated with disease resistance.
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Interaction transcriptome studies are a popular method for understanding responses of both the pathogen and the host during the infection process (Mosquera et al., 2009; Kim et al., 2010). In the past decades, enormous progress has been made in our understanding of pathosystems involving necrotrophic fungal species. However, our understanding of the molecular and biochemical mechanisms mediating plant infection by necrotrophic fungi is still very limited.

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 reports the quantitative expression of known defense genes in rice leaves upon B. oryzae infection.
4.2. Materials and Methods

4.2.1. Plant Materials and Growth Conditions

Rice (*Oryza sativa*) varieties used in this work included Jaya, Pushpa, IR 64, IET 13901 and KMP 101. The seeds were surface sterilized with 2% sodium hypochlorite solution for 2 min, rinsed three times in sterile distilled water, and germinated on wet sterile filter paper in sealed petri dishes (92% or greater relative humidity) at 28 °C. Five days later, the seedlings were grown in commercial potting soil under greenhouse conditions (30 ± 4 °C, 16h light/8h dark regime). Five-week-old plants (six- to seven-leaf stage) were used for infection with *Bipolaris oryzae*.

4.2.2. Pathogen Inoculation and Disease Rating

*Bipolaris oryzae* strain ‘BoKar’, isolated from the rice seeds (variety *Jaya*), 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, the 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 (6-7 leaf stage) were misted with *B. oryzae* conidial suspension (1mL per plant) using a sprayer. Immediately following inoculation, plants were moved into a dew chamber (30 ± 4 °C, 92% or greater relative humidity) to facilitate fungal penetration and, 18 h later, transferred to greenhouse conditions for disease development. Control plants were treated with sterile distilled water instead of pathogen inoculum. Disease symptoms were scored at 4 d after inoculation, and disease ratings were expressed on the basis of diseased leaf area and lesion type using a I to V disease severity scale: I, no infection or less than 2% of leaf area infected with small brown specks less than 1mm in diameter; II, less than 10% of leaf area infected with brown spot lesions with gray to white centres, about 1 to 3 mm in diameter; III, average of about 25% of leaf area infected with brown spot lesions with gray to white centers, about 1 to 3 mm in diameter; IV, average of about 50% of leaf area infected with typical spindle shaped lesions, 3mm or longer with necrotic gray centre and water-soaked or reddish brown margins, with little or no coalescence of lesions; V, more than 75% of leaf area infected with
coalescing spindle-shaped lesions (De Vleesschauwer et al., 2010). All infection trials were repeated at least twice with similar results. Based on disease severity scale, resistant and susceptible plants were selected and were used for further studies.

4.2.3. Change in defense enzyme activity

Leaf samples from control and infected samples were harvested at 0, 1, 2, 3, 4 and 5 days post-inoculation and frozen at -80 °C before further processing. Frozen leaf samples were crushed to a fine powder in a mortar under liquid nitrogen. Soluble proteins were extracted by resuspending the powder in four volumes of 50 mM sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM PMSF, 5mM sodium ascorbate, and 5% (wt/vol) polyvinyl-polypyrrolidone. The homogenate was centrifuged at 17,000 × g for 10 min. The supernatant was divided into aliquots, frozen in liquid nitrogen, and stored at -80 °C for further analysis. All of the above operations were carried out at 0 to 4 °C. Activity levels of POX and PAL in leaf extracts were measured spectrophotometrically.

4.2.3.1. Determination of peroxidase (POX) activity

Leaf samples (1 g) maintained at -80 °C were homogenized in 2 ml of 0.1 M phosphate buffer, pH 7.0 at 4 °C. The homogenate was centrifuged at 16, 000 g at 4 °C for 15 min and the supernatant was used as enzyme source. The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1% H₂O₂. The reaction mixture was incubated at room temperature (28 ± 2 °C). The changes in the absorbance at 420 nm were recorded at 30 s interval for 3 min. the enzyme activity was expressed as changes in the absorbance min⁻¹ mg⁻¹ protein (Hammerschmidt et al., 1982).

4.2.3.2. Determination of phenylalanine ammonia lyase (PAL) activity

Seed samples (1 g) stored at -80 °C were homogenized in 3 ml of ice cold 0.1 M sodium borate buffer, pH 7.0 containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinyl pyrrolidine. The extract was centrifuged at 16, 000 g for 15 min. the supernatant was used as enzyme source. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm as described by Dickerson et al. (1984). A sample containing 0.4 ml of enzyme extract was incubated
with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 min at 30 °C. The amount of trans-cinnamic acid synthesized was calculated using its extinction coefficient of 630 m\(^{-1}\) (Dickerson et al., 1984). Enzyme activity was expressed as nmol trans-cinnamic acid min\(^{-1}\) mg\(^{-1}\) protein.

### 4.2.3.3. Data analysis

Experiment was conducted in triplicates for each sample unless otherwise mentioned. Statistical analysis was performed with SPSS10.0 software for multiple comparisons and correlation analyses. A value of P<0.05 was considered to be statistically significant.

### 4.2.4. Genomic DNA extraction

Leaf samples from control and inoculated samples (from resistant and susceptible plants) were harvested at 4 dpi, 6 dpi, 8 dpi and 10 dpi and frozen at -80 °C before further processing. One gram of frozen leaf samples were crushed to a fine powder in a mortar under liquid nitrogen, and genomic DNA was extracted using a DNeasy Plant Mini kit (Qiagen, Bangalore, India) following instructions of the manufacturer.

#### 4.2.4.1. Real-time PCR assay for B. oryzae DNA quantification in infected rice

Real-time qRT-PCR was performed in 96-well plates using the LightCycler 480 (Roche, Hyderabad) and SYBR Green PCR master mix (Roche) according to the manufacturer’s instructions. The primer pairs specific to ITS2 of *B. oryzae* (BoVf 5’-CGCCCATCTTTATGCAGTTTCC-3’ and BoVr 5’-CGAGTCTCCAGAAAGAGG-3’) were used. The data was normalized with the DNA amount of a rice actin gene (AK060893), which was quantified using the forward, 5’-GAGTATGATGAGTCCGGTGTCAG-3’ and reverse, 5’-ACACCAACAATCCTCCAAACAGAG-3’. Amplification reactions were done in 20 µl volumes containing 10 pmol of each primer, 2 µl *B. oryzae* DNA, and 10µl 2X SYBR Green PCR master mix to detect dsDNA synthesis.

The thermal profile used consisted of an initial denaturation step of 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and 72 °C for 10 s to avoid dimer formation. Fluorescence signals were
collected at each polymerization step. The Ct values, upon which the calculations for relative expression levels of ITS2 and RAG were based, were the means of triplicate independent PCRs for both ITS2 and the endogenous control. No-template controls were included for each primer pair and each PCR reaction was completed in triplicate. Following amplification, a melting curve (MC) analysis (dissociation curve analysis) was performed for each amplicon to verify the specificity of each amplification reaction. The melting curve was obtained by heating the amplicon from 72 to 95°C with a heating rate of 0.1°C/s. The thermal profile of each reaction ended with cooling the amplicon to 40 °C for 10 s. Relative gene expression of genes was calculated by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) using the equation $\Delta\Delta C_T = (C_{T,\text{Target}} - C_{T,\text{Actin}})_{\text{Time}} - (C_{T,\text{Target}} - C_{T,\text{Actin}})_{0}$, where x represents the time points of 1, 2, 3, 4 and 5 h and 1, 2, 3, 4 and 5 days after B. oryzae infection.

4.2.5. RNA extraction

RNA was extracted from rice leaves using RNeasy Plant Mini kit (Qiagen, France) according to the manufacturer’s instruction and stored at -80 °C. The quality and quantity of RNA was deterimed with a Nanodrop ND-100 Spectrophotometer (Nanodrop Technologies, Germany). RNA quality was analyzed under UV illumination for the presence of distinct ribosomal bands and for the absence of degraded non-denaturing conditions on a 1% (w/v) agarose gel. DNase treatment of RNA was performed by the addition of 1µl RNase-free DNase (1 U/µl) (Fermenatas Life Sciences, Bangalore), 1 µl 10X reaction buffer with MgCl2, 1 µg RNA and upto 9 µl nuclease free water (Genei, Bangalore). The mixture was incubated at 37 °C for 30 min followed by the addition of 1 µl 25 mM EDTA and an incubation step of 65 °C for 10 min. DNase-treated RNA was column purified using RNeasy MiniElute Cleanup kit (Qiagen, France) according to manufacturer’s instruction. Quantification of the RNA content was done by diluting the samples with RNase-free water. Absorbance of the samples was measured at 260 and 280 nm.

4.2.5.1. First strand cDNA synthesis

First strand cDNA synthesis was carried out in an 11.6 µl reaction volume by adding 1 µl oligo(dT)$_{18}$ primers (Fermentas), 0.5 µl RNase inhibitor (40 U/ µl) (Fermentas) and RNase free water to 1 µg RNA. The mixture was incubated at 70 °C for 1 min and chilled on ice for 5 min. this was followed by the addition of 1 µl 10
mM dNTPs (Fermentas), 2.4 µl 15 mM MgCl₂, 4 µl 5X reaction buffer and 1 µl MMulv reverse transcriptase (Fermentas). This mixture was incubated at 25 °C for 10 min, 42 °C for 60 min and 70 °C for 15 min.

4.2.5.2. Reverse transcription PCR (RT-PCR)

PCR amplifications were carried out using first strand cDNA as the template. The PCR was conducted in a 20 µl reaction volume that contained 1.5 µl 15 mM MgCl₂, 2.5 µl 10 X buffer, 2 µl 2.5 mM dNTPs, 0.4 µl of each primer (10 µM), 0.5 U Taq polymerase (Genei, Bangalore), 0.4 µl DNA and nuclease free water. The concentration of the synthesized cDNA was determined, and the cDNA was diluted up to 0.75 - 0.85 µg/µl for use in RT-PCR. POX and PAL primers were obtained from Gomez-Ariza et al. (2007) and MPK primers were obtained from Reyna and Yang, 2006. RAG was designed and synthesized primers in the present study. The primer pairs used in the study are listed in Table 4.1.

Table 4.1. Primers used in real-time reverse transcription-PCR (qRT-PCR) for amplifying defense-associated genes in rice.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Designation</th>
<th>Oligo sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAL</td>
<td>OsPALF</td>
<td>Forward</td>
<td>TTCTATACAACAACGGGCTTCC</td>
<td>400 bp</td>
</tr>
<tr>
<td></td>
<td>OsPALR</td>
<td>Reverse</td>
<td>CCTGGAGGAAGATGAGACCAA</td>
<td></td>
</tr>
<tr>
<td>POX</td>
<td>OsPOXF</td>
<td>Forward</td>
<td>CGACATCCTGCAGTCGCC</td>
<td>250 bp</td>
</tr>
<tr>
<td></td>
<td>OsPOXR</td>
<td>Reverse</td>
<td>CTGTCCCTGAAGTCTGAGTGCAC</td>
<td></td>
</tr>
<tr>
<td>MAPK5</td>
<td>OsMAPK5F</td>
<td>Forward</td>
<td>ACGAGGACAAATGAAGACGC</td>
<td>205bp</td>
</tr>
<tr>
<td></td>
<td>OsMAPK5R</td>
<td>Reverse</td>
<td>AGCAGCCACAATCTGGAGAGA</td>
<td></td>
</tr>
<tr>
<td>MAPK7</td>
<td>OsMAPK7F</td>
<td>Forward</td>
<td>TCATCTGGAGGAATCCTTG</td>
<td>165bp</td>
</tr>
<tr>
<td></td>
<td>OsMAPK7R</td>
<td>Reverse</td>
<td>TGTCATTCGCCCCAACAAGT</td>
<td></td>
</tr>
<tr>
<td>MAPK12</td>
<td>OsMAPK12F</td>
<td>Forward</td>
<td>TCCAAGTACACACAGCATATTG</td>
<td>148bp</td>
</tr>
<tr>
<td></td>
<td>OsMAPK12R</td>
<td>Reverse</td>
<td>ATAGCATCTAAGGGGGGGTTTC</td>
<td></td>
</tr>
<tr>
<td>Rice actin</td>
<td>OsRAGF</td>
<td>Forward</td>
<td>GAGTATGATGAGTCGGGTCCAG</td>
<td>300bp</td>
</tr>
<tr>
<td></td>
<td>OsRAGR</td>
<td>Reverse</td>
<td>ACACCAAATCCCCAACAAGAG</td>
<td></td>
</tr>
</tbody>
</table>

RT-PCR was conducted in an Eppendorf Mastercycler gradient PCR Machine (Eppendorf Scientific, Germany). The standardized PCR conditions were as follows: 94 °C for 3 min, initial denaturation; 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min and a final extension of 72 °C for 10 min. Rice actin (housekeeping)
gene was used as an internal control in all reactions. In all RT-PCR runs, appropriate negative controls containing no cDNA template were subjected to the same procedure in order to exclude or detect possible contamination or carryover.

The RT-PCR products for all target genes were purified using QIAquick PCR Purification Kit and quantified using Nanodrop. Depending on the gene, serial dilutions of purified RT-PCR products of the range of concentration from $10^1$-$10^9$ copies and over 4 to 5 orders of magnitude were prepared to generate standard curves for real-time qRT-PCR assays.

### 4.2.5.3. Real-time quantitative PCR (qRT-PCR) and rice defense gene expression

Real-time qRT-PCR was performed in 96-well plates using the LightCycler 480 (Roche, Hyderabad) and SYBR Green PCR master mix (Roche) according to the manufacturer’s instructions. The primer pairs listed in Table 4.1. were used to amplify the corresponding 5 genes of interest during rice-\textit{B. oryzae} interaction. Amplification reactions and thermal profiles were same as mentioned earlier in section. The Ct values, upon which the calculations for relative expression levels of POX, PAL, MAPK5, MAPK7, MAPK12 and RAG were based, were the means of triplicate independent PCRs for both the target and the endogenous control. No-template controls were included for each primer pair and each PCR reaction was completed in triplicate. Relative gene expression of genes was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the equation $\Delta\Delta Ct = (C_{T,\text{Target}} - C_{T,\text{Actin}})_{\text{Time x}} - (C_{T,\text{Target}} - C_{T,\text{Actin}})_{\text{Time 0}}$, where x represents the time points of 1, 2,3,4 and 5 days for \textit{B. oryzae} infection.
4.3. Results

4.3.1. Green house screening of rice varieties against brown spot of rice

The disease reaction was categorized on I-V scale based on the severity (De Vleesschauwer et al., 2010). Rice varieties were grouped as resistant, moderately resistant, susceptible, moderately susceptible and highly susceptible. No varieties were highly resistant (Table 4.2). Brown spot severity in moderately resistant and highly susceptible rice varieties is shown in fig 4.1. For ease the moderately resistant and highly susceptible varieties will be mentioned in the following document as resistant and susceptible varieties.

Table 4.2. Screening of rice genotypes for brown spot disease severity

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Rice varieties</th>
<th>Disease severity scale I-V</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>IET 13901</td>
<td>II</td>
<td>Moderately resistant</td>
</tr>
<tr>
<td>2.</td>
<td>Pushpa</td>
<td>III</td>
<td>Moderately susceptible</td>
</tr>
<tr>
<td>3.</td>
<td>IR 64</td>
<td>III</td>
<td>Moderately susceptible</td>
</tr>
<tr>
<td>4.</td>
<td>KMP101</td>
<td>IV</td>
<td>Susceptible</td>
</tr>
<tr>
<td>5.</td>
<td>Jaya</td>
<td>V</td>
<td>Highly susceptible</td>
</tr>
</tbody>
</table>

Figure 4.1. Brown spot severity in two different rice genotypes. Disease symptoms were scored at 4 d after inoculation, and disease ratings were expressed on the basis of diseased leaf area and lesion type using a I to V disease severity scale. Plants were categorized as resistant and susceptible to brown spot based on disease severity scale.
4.3.2.1. Estimation of Phenylalanine ammonia lyase enzyme in resistant and susceptible rice varieties inoculated with *B. oryzae*

PAL activity was estimated at different time points before and after *B. oryzae* inoculation. In resistant control samples no considerable difference in PAL activity at different time points was observed. However, after 1 day inoculation increase in PAL activity was detected and higher activity was recorded at 4th day with 2.93 units increase in activity compared to control. In highly susceptible control, the activity was less and slightly changed at different time intervals (Fig. 4.2). The constitutive level of PAL activity was higher in resistant than in highly susceptible variety. In susceptible plants the activity peaked by 1.12 units on 2nd day and decreased to 0.67 units on 3rd day. The activity of PAL peaked again on 4th day with 0.88 units and activity dropped again on 5th day. It increased by 2.4 fold at 5 day compared to its control and gradually decreased thereafter at later time points. However the PAL activity in uninoculated and inoculated resistant variety was higher than in inoculated and uninoculated highly susceptible varieties.

**Figure 4.2.** Phenylalanine ammonia lyase (PAL) activity in resistant and susceptible rice leaves collected at different time intervals (0, 1, 2, 3, 4 and 5 days) after inoculation with *B. oryzae*. Resistant inoculated sample showed gradual increase in PAL activity and recorded maximum activity at 4th day and was higher compared to susceptible inoculated variety. In susceptible variety, PAL activity was less, compared to resistant inoculated and maximum activity was recorded on 3rd day post inoculation. Resistant control and susceptible control showed minimum activity compared to its respective inoculated samples. RC- Resistant control; RI-Resistant inoculated; SC-Susceptible control; SI-Susceptible inoculated.
4.3.2.2. Estimation of Peroxidase activity in resistant and susceptible rice varieties inoculated with *B. oryzae*

Resistant IET 13901 and susceptible Jaya rice varieties were inoculated with *B. oryzae* and POX activity was estimated at 0, 1, 2, 3, 4 and 5 days post-inoculation. Higher POX activity was recorded in resistant (IET 13901) variety when compared to highly susceptible (Jaya) variety (Fig. 4.3).

![Figure 4.3](image_url)

**Figure 4.3.** Peroxidase (POX) activity in resistant and susceptible rice leaves collected at different time intervals (0, 1, 2, 3, 4 and 5 days) after inoculation with *B. oryzae*. Resistant inoculated sample showed gradual increase in POX activity and recorded maximum activity on 3rd day and gradually declined thereafter. POX activity in resistant inoculated sample was higher compared to susceptible inoculated variety. In susceptible variety, POX activity was less, compared to resistant inoculated and maximum activity was recorded on 3rd day post-inoculation. Resistant control and susceptible control showed minimum activity compared to its respective inoculated samples. RC- Resistant control; RI-Resistant inoculated; SC-Susceptible control; SI-Susceptible inoculated.
4.3.3. Real-time PCR assay for *B. oryzae* DNA quantification in infected rice

Fungal DNA was quantified 4 days after *B. oryzae* infection using *B. oryzae* specific ITS2 primers in real time PCR analysis. The levels of *B. oryzae* in resistant and susceptible plants were quantified at 4, 6, 8, 10 and 12 dpi. DNA quantification in both genotypes was detected in real time PCR analysis. Control plants irrespective of genotype showed no amplification of fungal DNA. 4.35-fold of fungal DNA was detected in resistant variety on 4 dpi, that gradually increased by 5.39-fold and peaked significantly by 28 fold on 8 dpi; and subsequently no significant increase in *B. oryzae* ITS was observed on 10 and 12 dpi with 29 and 32 fold. In contrast, compared to resistant variety the susceptible variety showed high amount of fungal DNA throughout the analysis period. On 4 dpi fungal DNA in susceptible plant was detected by 47 fold, which increased on 6 dpi with 69 fold. Fold change significantly peaked by 122 fold on 8 dpi and further increased on 10 and 12 dpi with 138 and 149 fold respectively (Fig. 4.4).

![Figure 4.4](image.png)

**Figure 4.4.** Relative quantification of *B. oryzae* ITS2 in susceptible and resistant plants. (X- Days post inoculation (dpi) by *B. oryzae*) and (Y- ng DNA per gram leaf tissue). Normalized quantification of fungal levels by calculating the ratio of fungal rDNA-ITS and rice actin. (dpi - days post-inoculation)
4.3.4. Isolation of total RNA from resistant and susceptible rice varieties inoculated with *B. oryzae*

Electrophoresis of isolated RNA on 1% agarose gel stained with SYBR Green I showed distinct 28 S and 18 S rRNA bands, indicating a good quality of total RNA. The absorbance ratio A260/A280 ranged from 1.9 to 2.3 indicating the purity of extracted RNA. The quality of RNA was further evaluated by reverse transcription of the first strand cDNAs and qPCR. The concentration of various sample cDNAs is more than 700 µg/µl.

4.3.4.1. Quantification of defense-associated gene expression in *B. oryzae* infected rice

After the confirmation of amplification of target genes in reverse transcription PCR (RT-PCR) based on the intensity of the amplicon (Table 4.3), the samples were used in real-time PCR. Considering that different genes are activated during the host-pathogen interaction, it was necessary to investigate the expression patterns of the rice genes activated by infection of *B. oryzae*. To evaluate the specificity of amplification product of the defense genes, the reverse-transcribed cDNAs of infected rice samples were amplified by SYBR Green qPCR assay. Appearance of single sharp peak for POX, PAL, MAPK5, MAPK7, MAPK12 and the reference gene RAG from the melting curves ascertain the absence of primer dimers and non-specific amplification products. Meanwhile, PCR amplification curves also show that amplification has good reproducibility in each sample (Fig. 4.5). Melt curve analysis provide specific melting temperature (Tm) for each gene. Tm was 83 °C for RAG, 86 °C for PAL, 89 °C for MPK12, 80 °C for POX, 81 °C for MPK5 and 82 °C for MPK7.
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Table 4.3. Gene expression analysis based on reverse transcription PCR (RT-PCR)

<table>
<thead>
<tr>
<th>Gene/Days</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Gene/Days</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>POX</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>POX</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>PAL</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>PAL</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>RAG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>RAG</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene/Hours</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Gene/Hours</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPK5</td>
<td>+</td>
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[Note: Fifteen-day-old rice shoots (resistant and susceptible) were sprayed until runoff with B. oryzae conidial suspension or the control solution, and RT-PCR (reverse transcription PCR) was executed on cDNA synthesized using RNA from leaf tissue at known time intervals. The expression levels of the selected genes and housekeeping gene (rice actin) in rice leaves of resistant and susceptible plants are shown in terms of amplicon intensity observed on agarose gel. (-) no gene expression, (+) - mRNA levels of gene detected, (++) high expression of genes, (+++) very high expression of genes.]

Figure 4.5. The melt curves of (from left to right) POX, MPK5, MPK7, RAG, PAL and MPK12 qPCR product.
4.3.4.2. Relative gene expression in *B. oryzae* infected resistant and susceptible rice plants

The rice varieties IET 13901 and Jaya were further investigated to determine the correlation between infection in rice leaf tissues and timing of transcript accumulation of five defense response genes encoding peroxidase, phenylalanine ammonia lyase, mitogen activated protein kinases namely MPK5, MPK7 and MPK12. In each of three experiments, rice plants were sprayed with *B. oryzae* spore suspension or water. One plant each was sampled from water-sprayed and *B. oryzae*-sprayed IET 13901 and Jaya at 0, 1, 2, 3, 4 and 5 dpi for POX and PAL analysis; whereas samples were harvested at an interval of 0, 1, 2, 3, 4 and 5 hpi for MPKs analysis.

### 4.3.4.2.1. Effect of *B. oryzae* infection on MPK5 expression in resistant and susceptible rice plants

MPK5 is one of the major genes involved in SA-synthesis pathway. MPK5 gene was downregulated in susceptible plants upon pathogen infection at all time intervals. The attenuation was maximum (5.92-fold reduction) early (1 hpi) upon *B. oryzae* infection that remained down regulated till 5 dpi. In resistant plants the mRNA levels of MPK5 were induced earlier at 1 hpi (1.52-fold increase), but there was no significant change in the expression of the gene till 4 hpi. However, a slight increase was observed on 5 hpi with 3.09-fold increase. The attenuation of this gene in susceptible plants subsequently reduced with time points (Fig. 4.6).

### 4.3.4.2.2. Effect of *Bipolaris oryzae* infection on MPK7 gene expression in resistant and susceptible rice plants

MPK7 is one of the major genes involved in JA-synthesis pathway. Upon *B. oryzae* infection, MPK7 gene was significantly attenuated in the resistant plants at 1 and 2 hpi, however the downregulation at 2 hpi (2.2-fold reduction) was less compared to the 1 hpi (4.36-fold reduction). In contrast, mRNA levels of MPK7 were induced at 1 hpi (1.69-fold) and gradually increased at 2 hpi (2.78-fold) in susceptible plants. On 3 hpi, both genotypes showed maximum fold change in MPK7 expression, however, the fold change in susceptible plant was significantly high than the resistant plants with 33.65 and 10.62 fold respectively and subsequently declined (Fig. 4.6)
4.3.4.2.3. Effect of *Bipolaris oryzae* infection on MPK12 expression in resistant and susceptible rice plants

MPK12 is one of the major genes involved in JA-synthesis pathway. In susceptible plants, the mRNA levels of MPK12 remained attenuated up on pathogen inoculation from 1 hpi to 4 hpi (3.74-fold reduction) and reached above basal expression level at 5 hpi with 1.89 fold increase. In resistant plants, MPK12 expression was induced on 1 hpi (1.72-fold) with significant increase observed at 3 hpi with 16.35-fold increase. The expression dropped to basal expression level at 4 hpi (1.23-fold) and peaked by 7.23-fold increase at 5 hpi in susceptible plants (Fig. 4.6)

4.3.4.2.4. Effect of *B. oryzae* infection on POX expression in resistant and susceptible rice plants

The expression of rice POX gene was evaluated in IET 13901 (MR) and Jaya (S) at different time intervals after *B. oryzae* infection. The expression levels of POX were lower in MR plants than in S plants after *B. oryzae* infection. There was a significant difference in gene expression between the two rice genotypes. Early upon infection with *B. oryzae*, POX gene was down regulated by 1.12-fold reduction in IET 13901 (MR) and returned to basal expression level on 2 dpi (1.26 fold). In Jaya (HS), POX expression was induced on 1 dpi with 1.5-fold increase. The relative expression of POX in Jaya (HS) was 1.92, 5.6, 7.62 and 5.22 times greater than that in IET 13901 (MR) at 2, 3, 4 and 5 dpi respectively. The expression level was substantially lower in IET 13901 (MR) at subsequent time points (Fig. 4.6)

4.3.4.2.5. Effect of *B. oryzae* infection on PAL expression in resistant and susceptible rice plants

Evaluation of the expression of the PAL gene after infection with *B. oryzae* established that time and variety had significant effect on the expression of PAL. Highest expression level of PAL gene was observed at 1 dpi in resistant genotype. The gene was also differentially expressed. In the 1-5 dpi analysis window, most of the significant changes in gene expression occurred between 1-2 dpi in PAL. The fold change in the expression of this gene was 3.92 times in resistant genotype IET 13901, whereas it was the least in Jaya (1.56). In resistant genotype the expression of this
gene was induced on 1 dpi and peaks to 3.92-fold on 2 dpi, whereas in susceptible plants, expression of this gene was induced at 2 dpi (1.56), subsequently decreased and reached basal level expression. The expression gradually declined on 3 dpi but maintained at higher levels (2.11-fold) on 5 dpi in resistant plants but was down regulated in susceptible plants (Fig. 4.6)

Figure 4.6. Relative fold expression of rice POX, PAL, MPK5, MPK7 and MPK12 genes due to B. oryzae infection. Rice seedlings were spray-inoculated with B. oryzae conidial suspension. Total RNAs were isolated from the leaf samples collected 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.
4.4. Discussion

To effectively combat invasion by microbial pathogens, plants have evolved sophisticated mechanisms providing several strategic layers of constitutive and induced defenses. These inducible defenses are regulated by coordinated activity of an elaborate matrix of signal transduction pathways in which the plant hormones SA, JA and ET act as key signaling molecules (Grant and Lamb, 2006; Adie et al., 2007). In response to pathogen attack, plants produce a highly specific blend of SA, JA and ET, resulting in the activation of distinct sets of defense-related genes (Koornneef and Pieterse, 2008). This signal-signature varies greatly in quantity, timing and composition in response to the type of attacker encountered; and plays a primary role in the orchestration of the plant’s defense response and eventually determines the specific nature of the defense response triggered (Vos et al., 2005).

The genes and enzymes involved in host-pathogen interactions have been extensively reviewed, and the selected genes in the study are involved in the first line of defense mechanism. The expression of all these genes was studied by qPCR in resistant and susceptible rice leaves.

The transcript level of PAL, MPK5 and MPK12 were significantly high in the R genotype than in S genotype. Accumulation of PAL, the key gene in the SA-dependent pathway was far more rapid in R genotype and its expression was induced by *B. oryzae* challenge inoculation. Similarly, MPK5 and MPK12 expression was significantly high in R plants upon *B. oryzae* inoculation. Early accumulation of MPK12 (3 hpi), followed by MPK5 (5 hpi) expression depicts their defensive role in rice-*B. oryzae* pathosystem. It is believed that activation of these two MPKs in series might have triggered PAL expression and thus, rice upon *B. oryzae* infection follows SA-mediated defense signaling pathway in R genotype. In contrast, accumulation of POX, a major gene involved in JA signaling pathway was much greater in S genotype than in R. On the other hand, fold change in MPK7 expression was high in S genotype. Interestingly, though MPK7 expression was highly induced in susceptible genotype, its expression was also considerably strong in the resistant genotype. This may be an indication that MPK7 triggers POX expression and follows JA-signaling pathway, whereas MPK5 and MPK12 triggers PAL expression and follows SA-signaling pathway. The expression of MPK7 in both R and S genotype indicates that
it may be involved in both JA and SA signaling pathway. However, the key role of these genes in rice defense against *B. oryzae* has to be confirmed by mutant analysis and RNA silencing.

In general, the SA-dependent signaling pathway regulates the expression of a wide array of defense response genes and confers broad-spectrum pathogen resistance (Jia *et al.*, 2000). Activation of the SA pathway has been proven to be important in both basal and resistance (R) gene-mediated biotrophic or hemibiotrophic pathogen defense, while JA/ET pathway is activated in response to necrotrophic pathogens (Pieterse *et al.*, 2009). Responses to infestation by necrotrophs vary with host and the type of attacker as previously mentioned and induces either JA or SA-dependent defenses. Plant mutants and transgenics have revealed that JA-dependent responses are predominantly effective and resulted in resistance to necrotrophic fungi *Alternaria alternata* and *Botrytis cinerea* (Thomma *et al.*, 1998). In contrast, JA mutants showed high mortality to *Fusarium* species (Thaler *et al.*, 2004). Apart from this, there are several examples that clearly point to a role of JAs in resistance against pathogen with diverse lifestyles, challenging the general notion that JA-dependent defense responses are predominantly effective against necrotrophic pathogens (Ellis *et al.*, 2002; Pozo *et al.*, 2005). On the other hand, infection with biotrophic pathogens induces SA-mediated defense responses resulting in increased susceptibility to necrotrophic pathogens by suppression of JA-signaling pathway (Spoel *et al.*, 2007).

Based on the present molecular investigation and in correlation with the previous cellular basis of analysis (De Vleesschauwer *et al.*, 2010), an assumption can be made that early stages of *B. oryzae* infection exhibit a short biotrophic phase that later colonize and feed on the host cells resulting in the necrotic stage. It can thus be believed that SA-dependent pathway played a key role during early biotrophic phase and JA-dependent pathway was activated during later stages of infection where *B. oryzae* entered the necrotic phase of life cycle. The gene expression analysis in rice-*B. oryzae* interaction in the present study provoked us to look for the effect on resistance to brown spot by exogenous application of JA and SA as inducers.