5.1. Introduction

Most plants have the ability to escape invasion of pathogens by using defence systems, even if they do not have a specific disease resistance gene. There is a delicate relationship between plant and pathogen. When environmental conditions such as temperature and humidity are favourable for the pathogen, the pathogen can easily invade the plant. When the defense system of the plant functions effectively on the other hand, the plant can overcome pathogen attack. In the absence of effective chemical or other control agents against the bacterial leaf blight pathogen, host resistance has gained enormous importance in controlling this disease. In rice, the genetics of resistance to several pathogens has been well characterized. Resistance of rice plants towards *X. oryzae* pv. *oryzae* at different growth stages varies according to host genotypes as seedling resistance (at seedling stage) and adult plant resistance (at adult stage but susceptible at seedling stage). In response to a pathogen, the host plant expresses various degrees of resistance which is usually classified into two categories namely qualitative resistance and quantitative resistance. Qualitative resistance is generally controlled by major genes while quantitative resistance is controlled by polygenic factors. Higher plants have a broad range of mechanisms to protect themselves against various threats including physical, chemical and biological stresses, such as wounding, exposures to salinity, drought, cold, heavy metals, air pollutants and ultraviolet rays and pathogen attacks, like fungi, bacteria and viruses. Plant reactions to these factors are very complex, and involve the activation of a set of genes, encoding different proteins. These stresses can induce biochemical and physiological changes in plants, such as physical strengthening of the cell wall through lignification, suberization and callose deposition by producing phenolic compounds, phytoalexins and pathogenesis-related (PR) proteins which subsequently prevent various pathogen invasions. Plants responds to bacterial pathogen attack by activating different defense responses leads to accumulation of defense enzymes, inhibitors and antibiotics etc. which prevents the pathogen infection. Plant pathogen interaction is mediated by the complex network of molecular and cytological events that determine a range between susceptibility and resistance.

Peroxidase is believed to be one of the most important factors of the plant biochemical defense against pathogenic microorganism, which is actively
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participating in the self regulation of plant metabolism after infection. Peroxidase involves a variety of plant defense mechanism in which hydrogen peroxidase is often supplied by an oxidative burst. Disease resistance in plants is associated with the activation of wide array of defense responses that prevents pathogen infection. Peroxidase induction appears to occur in both compatible and incompatible interaction in many host-pathogen interactions. Peroxidases, ascorbate peroxidases (APX, EC 1.11.1.11) and glutathione peroxidase (GPX, EC 1.11.1.9) which uses ascorbate and glutathione as electron donors, respectively, are well known for their role in H$_2$O$_2$ detoxification in plant (Chandrashekar and Umesha, 2012). Polyphenol oxidase is a nuclear encoded, plastid copper-containing enzyme which catalyses the oxygen-dependant oxidation of phenols to quinines. Because of conspicuous reaction products and induction by wounding and pathogen attack. There are few reports of the role of polyphenol oxidase in plant defense against pathogens.

5.2. Materials and methods

5.2.1. Seed sample and microorganism

Ten different rice cultivars were procured from private seed agencies, Mysore and Mandya, India. All seed samples were surface sterilized with 1% (v/v) sodium hypochlorite solution for 4 min and were thoroughly washed with distilled water three times and surface dried. The species specific PCR confirmed culture of Xanthomonas oryzae pv. oryzae (Xoo-05) was selected for further study.

5.2.2. Temporal Pattern Study of Enzymes

Ten rice cultivars were plated in Petri dishes onto moist blotter discs, at a density of 25 seeds per plate following standard procedures of the International Seed Testing Association (ISTA, 2005). The plates were incubated at 28±2°C for eight days and were used for further experiments. X. oryzae pv. oryzae inoculum was prepared as explained previously in the chapter IV and 8-day-old rice seedlings were covered with polythene sheeting 2 h before inoculation to increase the humidity. The rice seedlings were root-dip and spray inoculated with the inoculums (1x10$^8$ cfu/ml) and kept covered with polythene sheeting. To study the temporal pattern of peroxidise and polyphenol oxidase enzymes, ten different cultivars of rice, that were resistant (cv. Rajdhan), susceptible (cv. IR 501) and highly susceptible (cv. Jaya) category based on
the disease incidence under greenhouse conditions were selected. The rice seedlings were raised as explained previously and seedlings were harvested at different time intervals: 0, 3, 6, 9, 12, 15, 18, 21, and 24 up to 72 h after pathogen inoculation (hpi). Distilled water inoculated samples served as control.

5.2.3. Estimation of peroxidase activity

The peroxidase (E. C. 1.1.1.17) activity was determined according to standard procedure (Hammerschmidt et al., 1982). One gram of the seedling was homogenized in two ml of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was used as an enzyme source. The enzyme extract (100 ml) was taken along with 1.5 ml of guaicol (0.05 M). To initiate the reaction, 100 ml of hydrogen peroxide (1%) (v/v) was added to the sample cuvette and the absorbance was read at 420 nm (Hitachi U-2000 Japan). The enzyme activity was expressed as change in absorbance min$^{-1}$ g$^{-1}$ fresh tissue.

5.2.4. Estimation of polyphenol oxidase

Polyphenol oxidase ((E.C.1.14.18.1; PPO). activity was determined as per the procedure given by Mayer et al. (1965). The freeze dried samples (1 g) were homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16,000 g for 15 min at 4°C. The supernatant was used as the enzyme source. The reaction mixture consisted of 200 μl of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5). To start the reaction 200 μl of 0.01 M catechol was added and the activity was expressed as changes in absorbance at 495 nm min$^{-1}$ mg$^{-1}$ protein.

5.2.5. Protein estimation

Protein contents of the extracts were determined according to Bradford (1976) using BSA (Sigma, St Louis, MO, USA) as the standard.

5.2.6. Statistical analysis

All experiments were performed three times with similar results. The data from greenhouse experiments were analyzed separately for each experiment using analysis of variance (ANOVA) in the statistical software sas (version 9.0; SAS
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Institute Inc., Cary, NC, USA) for Microsoft Windows. Significant effects of pathogen inoculation on enzyme activities were determined by the magnitude of the F-value (P < 0.05). The mean values were compared for significance using Fisher’s LSD.

5.3. Results

5.3.1. Temporal changes in POX activity

The temporal changes in POX activity of resistant (R), susceptible (S) and highly susceptible (HS) seedlings with or without pathogen inoculation are shown in figure (Figure 5.1). Varying patterns of POX activity were observed in inoculated and control seedlings in R, S and HS cultivars. A gradual increase in POX activity was observed in all type of cultivars. In resistant seedlings, after pathogen inoculation a drastic increase in POX activity was noticed initially and reached its peak at 12 h (51.8 units) mean while, in S and HS seedlings, POX activity was found to be 28 and 25 units, respectively. Highest POX activity in S and HS seedlings was found at 36 h (31.4 units) and 42 h (25 units) respectively after pathogen inoculation (Figure 5.1).
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Figure 5.1 Temporal pattern study of POX activity in resistant (R), susceptible (S) and highly susceptible (HS) rice cultivars with and without *X. oryzae* pv. *oryzae* inoculation. The data expressed as the average of three independent experiments with three replicates each. Bars indicate standard errors.

5.3.2. Temporal changes in PPO activities

The temporal pattern was used to estimate the PPO peak activities of cultivars of different category of resistance upon pathogen inoculation. The samples were harvested at different time intervals from 0–72 hpi. There was a significant (P≤0.05) change in PPO activities in all seedlings after pathogen inoculation compared to untreated seedlings. PPO activity increased after pathogen inoculation. In resistant seedlings, the PPO activity reached its maximum at 21 hpi (32 units) and declined thereafter (6 units). Highest PPO activity in S and HS seedlings was found at 36 h (14 units) and 48 h (6 units) respectively after pathogen inoculation (Figure 5.2).
Figure 5.2 Temporal pattern study of PPO activity in resistant (R), susceptible (S) and highly susceptible (HS) rice cultivars with and without X. oryzae pv. oryzae inoculation. The data expressed as the average of three independent experiments with three replicates each. Bars indicate standard errors.

5.3.3. Spectrophotometric estimation of POX enzymes for different rice cultivars

Seedlings of 10 different rice cultivars were analysed for POX with or without pathogen inoculation. All the cultivars showed an increased level of enzyme activity after pathogen inoculation. Highest POX activity of 38 units was observed in Rajadhan cultivar followed by Arabisona and IR501, whereas least activity of POX was found in cultivar Thanu (21 units) after pathogen inoculation showing significant (p≤0.05) difference between their respective controls. A moderate level of increased POX activity was found in the cultivar PHB (25.6 units) after pathogen inoculation (Figure 5.3).
5.3.4. Spectrophotometric estimation of PPO enzymes for different rice cultivars

All the rice cultivars had showed increased PPO activities after pathogen inoculation. There were varied levels of PPO activity between cultivars. The highest PPO activity was in cv. Rajadhan (24 OD at 234 nm/mg protein/min) upon pathogen inoculation, a 3-fold increase when compared to control (6 OD at 234 nm/mg protein/min) and other cultivars. The least activity was in cv. Jaya (9 OD at 470 nm/mg protein/min) seedlings upon pathogen inoculation compared to control (5 OD at 470 nm/mg protein/min) A moderate level of increased PPO activity was found in the cultivar PHB (13 OD at 234 nm/mg protein/min) after pathogen inoculation (Figure 5.4).
Figure 5.4  PPO activity in different rice cultivars at 21 h with and without *X. oryzae* pv. *oryzae* inoculation. The data expressed as the average of three independent experiments with three replicates each. Bars indicate standard errors.
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5.4. Discussion

The present study was investigated in an intact host pathogen interaction between rice and *X. oryzae* pv. *oryzae*. Ten rice cultivars used in this study clearly varied in the degree of host resistance to the pathogen. Infection by pathogens is one of the major stress stimuli that plants encounter often. In response to the infection, the host produces a number of pathogen inducible enzymes, which are implicated in defense against phytopathogens. Early and elevated levels of expressions of various defense enzymes are an important feature of plant resistance to pathogens. Plants have their own enzymatic resources such as PAL, PPO, POX, LOX and \( \beta \)-1,3-glucanase. Peroxidases are the abundant enzymes which contain iron as heme and are responsible for both scavenging of \( \text{H}_2\text{O}_2 \) by the oxidation of phenols and its generation, through the oxidation of NADH. The induction of POX appears to be an early event in plant-microbe interactions. The correlation between increased POX activity during incompatible interactions and reinforcement of cell walls with phenol compounds was also reported (Polle *et al.*, 1994; Otter and Polle 1997; Shi *et al.*, 2007).

In the present study, an attempt has been made to study the resistance and susceptibility to different rice cultivars against *X. oryzae* pv. *oryzae* by considering POX and PPO as biochemical markers. Out of the 10 different rice cultivars evaluated potential sources of resistance to *X. oryzae* pv. *oryzae* only Rajadhan cultivar showed minimum disease incidence. None of the rice cultivars were free from leaf blight symptoms after pathogen inoculation and the susceptibility of cultivars was highest. In the present study, we report the direct involvement of POX and PPO during host-pathogen interaction in rice when inoculated with *X. oryzae* pv. *oryzae*. Spectrophotometric assays of POX and PPO enzymes in resistant, susceptible and highly susceptible seedlings without pathogen inoculation recorded lesser activities compared with inoculated seedlings, indicating a possible role of these enzymes during pathogen infection and host-resistance. The fact that POX and PPO activity was higher in resistant seedlings than in susceptible and highly susceptible seedlings indicates that POX and PPO might have played a specific role in triggering the development of host resistance. Our findings are similar with the earlier studies of
Chittoor et al. (1997) where they have studied the role of POX in resistant interactions between rice and *Xanthomonas oryzae pv. oryzae* which causes bacterial blight disease. Increased POX activity was observed in both resistant and susceptible interactions in rice. In the resistant cultivars, the activity of POX was higher when compared to the uninoculated seedlings and also with the susceptible and highly susceptible cultivars. In rice, LOX activity has been described to correlate positively with resistance to blast disease, since the octadecanoid pathway is activated after infection by the fungus. However, high LOX activity may constitute in plants resistant to pathogens but with an additional increase upon infection. (Sandhu et al., 2007). Evaluated the rice lines resistance to blast disease and LOX activity of non induced and induced plants with methylejasmonic acid. They reported that the resistance was associated with the enzyme activity, as high activity was strongly associated with disease resistance.

POX activity was noted to increase significantly in both resistant and susceptible cultivars as compared with their parent. These results are in agreement with other studies, which reported that increase in peroxidise activity enhance lignifications in response to chocolate spot infection which may restrict the fungal penetration. These findings indicate to a positive relationship between resistance and peroxidase activity. Peroxidase also produces free radicals and hydrogen peroxide which are toxic to many microorganisms. Another supportive suggestion was brought by Mohammadi and kazemi, (2002) who found that, a significant increase in POX specific activity in both resistant (Wang-shuibai) and susceptible (Falat) wheat cultivars following the inoculation with *Fusarium graminearum* conidia. Also, an increase in peroxidase activity is considered as a preliminary indicator for resistance of broad beans to chocolate spot disease (Nawar and Kuti, 2003). PPO specific activity significantly increased in wheat heads of resistant and susceptible cultivars following the inoculation with *F. graminearum* conidia. Similar results have been obtained in plant–pathogenic fungal interactions such as cabbage /*F. oxysporum*, onion/Botrytis, sunflower/Sclerotinia sclerotiorum, soybean/Phytophthora megasperma and bean /Rhizoctonia In addition, pretreatment of Chick pea with rhizobium increased significantly the levels of peroxidase and polyphenyl oxidase and total phenolics was recorded (Arfaoui et al., 2005). POX and PPO are important in the defense mechanism against pathogens, through its role in the oxidation of phenolic
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compounds to quinines, causing increasing in antimicrobial activity. Therefore, it may be involved in stopping pathogen development (Shimzu et al., 2006; Melo et al., 2006) accelerating the cellular death of cells close to the infection site, preventing the advance of infection and/or by generating a toxic environment which will inhibit the growth of the pathogen inside the cells (Naglaa et al., 2011).

Although the physiological function of the PPO in plants remains unclear because of their conspicuous reaction products and their wound and pathogen inducibilities. PPO have frequently been suggested to participate in plant defense against pathogen. Our studies reveals that PPO is involved during development of resistance by rice to bacterial leaf blight disease. The temporal pattern study of PPO activity determined with and without pathogen inoculation showed that in inoculated seedling the activity was maximum at 21hpi when compare to its control suggesting that PPO might be playing a specific role in triggering host resistance.

Our studies indicate that the defense enzymes POX and PPO are actively involved in imparting resistance to bacterial blight of rice. It was observed that upon inoculation of resistant seedlings, POX and PPO expression were elevated. This may inhibit the growth of pathogen by suppressing attempted invasion there by imparting resistance to bacterial blight of rice. From these studies it appears that POX and PPO are involved in imparting resistance to bacterial blight of rice. Further, POX and PPO can be used as a biochemical markers to indicate the resistance/susceptibility nature of rice cultivars against bacterial blight disease of rice.