CHAPTER 2

Elicitation of Defense Responses by Elicitors of PGPF- *Penicillium chrysogenum* in Pearl Millet against Downy Mildew Disease
CHAPTER 2

Introduction

A large number of strategies have been developed to overcome the problems caused by plant diseases and one of them is being the exploitation of targeted use of defense mechanisms that are inherent to plants (Hammerschmidt and Kuc, 1995; Kuc, 2001). In addition, it has been shown that plants can recognize general structures associated with microorganisms, so-called elicitors (Sharp et al., 1984; Strobel et al., 1996; Felix et al., 1999; Dong et al., 1999; Nurnberger and Brunner, 2002; Aziz et al., 2003). Elicitors after binding with specific receptors of plants, results in mounting elicitation of complex defense mechanisms (biochemical and mechanical) against pathogens (Linthorst, 1991; Benhamou et al., 1996; van Loon; 1999; Mansfield, 2000; Bonas and Lahaye, 2002; Nurnberger and Brunner, 2002). It has been shown that depending on the stimulus, specific signal transduction pathways involving one or several key regulators are activated leading to resistance against specific sets of pathogens (Tamm et al., 2011). The application of an elicitor treatment to plants may trigger defense mechanisms either directly or indirectly upon pathogen attack (Walters and Boyle, 2005).

Induction of systemic resistance (either local or systemic) is associated with expression of defense related genes (Conrath et al., 2001), production of antimicrobial compounds like phytoalexins (Mansfield, 2000), deposition of lignin (Anterola and Lewis, 2002) and/or callose (Yun et al., 2006), increased activities of a number of enzymes (Nicholson and Hammerschmidt, 1992) together eventually resulting in the development of pathogen and finally resulting in stronger elicitation of resistance or indeed, other defenses following pathogen attack (Hammerschmidt and Nicholson, 1999; Conrath et al., 2001; Kuc, 2006). Induction of resistance can be achieved by treating susceptible plants with different elicitors/inducers like PGPR (Chowdappa et al., 2013), PGPF (Hyakumachi, 1994; Meera et al., 1994; Koike et al., 2001; Hossain et al., 2007; Nagaraju et al., 2012), plant extracts (Baysal and Zellar, 2004; Chandrashekara et al., 2007; Shivakumar et al., 2009; Mythrashree et al., 2013) and non-pathogenic microorganisms (Howell et al., 2000; Killic-Ekici and Yuen, 2003).
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Introduction

Induction of defense related proteins make the plant resistant to pathogen invasion (van Loon, 1997). There are number of reports on these defense proteins correlated with defense against plant pathogens in various crop plants like chilli (Naveen et al., 2013), pearl millet (Nandini et al., 2013), tobacco (Beaudoin-Eagan and Thorpe, 1985) and cucumber (Rasmussen, 1991). Deposition of lignin and callose is also reported as one of the mechanisms in defense (Chalfoun et al., 2011). Increased levels of POX and lignin have been reported in cotton upon treatment with killed Penicillium (Dong et al., 2003) against Verticillium wilt disease. A novel hypersensitive response-inducing protein elicitor isolated from Magnaporthe oryzae induced callose deposition upon treatment to rice plants against M. oryzae (Chen et al., 2012).

In pearl millet there are number of reports on defense related enzymes involved in downy mildew disease resistance (Amruthesh et al., 2005; Geetha et al., 2005; Niranjanraj et al., 2006; Shivakumar et al., 2009; Sudisha et al., 2011; Mythrashree et al., 2013). There are also reports on deposition of lignin and callose during induction of resistance against the same pathogen (Hindumathy et al., 2006; Niranjanraj et al., 2012). However, there are only a few reports on the use of PGPF elicitors for pearl millet downy mildew disease management. Hence, present study was intended to investigate the efficacy of PGPF elicitors on induction of histological and biochemical changes during defense against downy mildew disease of pearl millet.

The results of first chapter have revealed that elicitor/ inducer [conidial suspension (1 x 10^8 cfu/ml) as well as CPE (at 10 µg/ml)] of PGPF- P. chrysogenum PGPF UOM 27 (PenC- JSB9) gave highest disease protection against downy mildew disease compared to all other PGPF isolates. Hence, in this chapter we intend to study the effect of elicitors/ inducers (conidial suspension and CPE) of PGPF - PenC- JSB9 on defense responses during pearl millet downy mildew host- pathogen interactions at morphological, histological and biochemical level.
Chapter 2  Materials and Methods

MATERIALS AND METHODS

Host plant

Seeds of pearl millet that are susceptible (7042S) and resistant (IP18292) to downy mildew disease were obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patencheru, India. The seeds were obtained under a Material Transfer Agreement and were used throughout the study.

Elicitor/ Inducer treatment

The pearl millet susceptible (7042S) and resistant (IP18292) seeds were surface sterilized with 0.02% sodium hypochlorite solution for 2 min and rinsed thoroughly in SDW 2-3 times. PGPF – PenC- JSB9 was used for seed treatment with conidial suspension (1 x 10^8 conidia/ ml) for 6 h and CPE (10 µg/ ml) for 3 h, respectively.

Sampling of seedlings

The treated seeds along with control were placed on Petri plates lined with moistened blotter discs and incubated at 25±2º C. Two-day-old seedlings were carefully removed without damaging the roots and dipped in spore suspension of pathogen at 4 x 10^4 zoospores/ ml.

The seedlings were harvested at 0, 3, 6, 9, 12, 24 h after inoculation (h.a.i) for hypersensitive reaction, lignin deposition and callose formation. For biochemical studies pearl millet seedlings were harvested at 0, 3, 6, 9, 12, 24, 48 and 72 h.a.i. and immediately stored at -80º C until used for further studies. Uninoculated pearl millet resistant, inducer treated and susceptible seeds served as control.

Morphological Studies

Time-course analysis for hypersensitive reaction (HR)

HR study was carried out in sampled seedlings following the method of Sharada (1995). The inoculated and uninoculated pearl millet seedlings were observed at hourly intervals for the external appearance of necrotic spots or streaks on the coleoptile region of tested seedlings. The initial time of appearance of HR and the number of seedlings showing the necrotic spots during the experimental period of 24
h were recorded and the percentage of HR showing seedlings were calculated. The experiment consisted of four replicates of 25 seedlings each and repeated three times. 

\[
\text{Per cent HR} = \frac{\text{No. of seedlings with necrotic spots}}{\text{Total no. of seedlings taken}} \times 100
\]

Histological Studies

**Time-course analysis for lignification**

Lignification study was carried out as described by Sherwood and Vance (1976). Epidermal peelings of inoculated and uninoculated pearl millet seedlings were placed in 2% phloroglucinol in 95% ethanol for 2 h. The tissues were then placed in a drop of 35% HCl on a slide and heated over a low-flame until the veins turned reddish purple. The slides were then observed under a microscope for the intensity of coloration and the cells were counted and percentage of lignified cells was calculated. The experiment consisted of four replicates of 25 seedlings each and repeated three times.

**Time-course analysis for callose deposition**

Epidermal peelings were placed in 0.005% water-soluble aniline blue in 0.15 M di-potassium hydrogen phosphate (pH 8.2) for 1 h and mounted in glycerol (Jensen, 1962). The epidermal peelings were then observed under fluorescence microscope where k = 365–405 nm. Region with callose deposition fluoresced. For microscopic observation 20 microscopic fields were counted for percentage calculation. The experiment consisted of four replicates of 25 seedlings each and repeated three times.

Biochemical studies

**Phenylalanine Ammonia Lyase (PAL) activity**

One gram fresh weight of seedlings harvested at above mentioned time intervals was homogenized in 1 ml of ice cold 25 mM Tris buffer, pH 8.8, containing 32 mM of 2- mercaptoethanol in a prechilled mortar and pestle. The extract was centrifuged at 10,000 rpm for 25 min at 4° C and the supernatant was used as enzyme.
source. Reaction mixture containing 0.5 ml of enzyme extract was incubated with 1 ml of 25 mM Tris- HCl buffer, pH 8.8 and 1.5 ml of 10 mM L- phenylalanine in the same buffer for 2 h at 40° C. The activity was stopped using 5 N HCl (Geetha et al., 2005). PAL activity was determined as the rate of conversion of L- phenylalanine to trans-cinnamic acid at 290 nm. Enzyme activity was expressed as µmol of trans-cinnamic acid/ mg protein/ h. Each experiment was repeated three times taking three replicates each time.

**Peroxidase (POX) activity**

One gram fresh weight of seedlings harvested at above mentioned time intervals was macerated with 0.2 M sodium phosphate buffer (pH 6.5) in a prechilled mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 15 min at 4° C to get the supernatant. POX activity was determined following the method of Hammerschmidt et al. (1982). The reaction mixture of 3 ml consisted of 0.25% (v/v) guaiacol in 10 mM potassium phosphate buffer (pH 6.9) containing 10 mM hydrogen peroxide. 5 µl of crude extract was added to initiate the reaction, which was followed colorimetrically at 470 nm. POX activity was expressed as the increase in absorbance at 470 nm/ mg protein/ min. Each experiment was repeated three times taking three replicates each time.

**β-1,3-Glucanase activity**

One gram fresh weight of seedlings harvested at above mentioned time intervals was homogenized in 0.05 M sodium acetate buffer (pH 5.2) and assayed according to the method of Kini et al. (2000) with 0.1% laminarin (Sigma) used as the substrate. Products released after incubation were estimated for reducing groups at 540 nm using the dinitrosalicylic acid reagent. Enzyme activity was expressed in terms of µmol/ min/ mg protein. Each experiment was repeated three times taking three replicates each time.

**Chitinase activity**

One gram fresh weight of seedlings harvested at above mentioned time intervals was macerated using 0.05 M sodium acetate buffer, pH 5.2 (1 ml/ g fresh
weight) and acid washed glass beads at 4° C in prechilled mortar and pestle. The homogenate was centrifuged at 10,000 rpm for 30 min at 4° C and the supernatant was used as crude enzyme extract. Chitinase was assayed following the method of Isaac and Gokhale (1982) with N-acetyl glucosamine (Sigma) as standard. Colloidal chitin in 0.05 M sodium acetate buffer (pH 5.2), purified from chitin (Sigma-Aldrich, USA) following the method of Skujins et al. (1965) was used as a substrate. Monomers of N-acetyl glucosamine released after incubation were measured spectrophotometrically at 585 nm using dimethyl amino benzaldehyde reagent (Reissig et al., 1955). The enzyme activity was expressed in terms of nmol/ min/ mg protein. Each experiment was repeated three times taking three replicates each time.

**Lipoxygenase activity (LOX)**

One gram fresh weight of the seedlings was homogenized in 0.2 M sodium phosphate buffer (pH 6.5) containing 1% polyvinylpyrrolidone (PVP), 0.1 % Triton X-100 and 0.04 % sodium meta-bisulfite. The homogenate was centrifuged at 9000 g for 20 min at 4° C and the supernatant was used as the enzyme source. Enzyme activity was measured by monitoring the appearance of the conjugated dienehydroperoxide at 234 nm. Linoleic acid was used as substrate, which was prepared according to the standard method (Axelrod et al., 1981). Activity was recorded for 3 min using a spectrophotometer. The enzyme activity was expresses in terms of mmol quinone formed/ min/ mg protein. Each experiment was repeated three times taking three replicates each time.

**Protein estimation**

Protein content in extracts was estimated by the dye binding method (Bradford, 1976) using bovine serum albumin (BSA) (Sigma) as a standard.

**Statistical analysis**

Each experimental data was subjected to analysis of variance (ANOVA) using SPSS Inc. 16.0. Significant effects of treatments were determined by the magnitude of the F value (P ≤ 0.05). Treatment means were separated by Tukey’s HSD test.
RESULTS

Morphological studies

Effect of PenC- JSB9 elicitors/inducers on hypersensitive reaction (HR)

While analyzing the effect of PenC- JSB9 inducers on the expression of defense reactions, a time course study on HR was conducted and recorded as described in materials and methods. Initial appearance of HR was recorded at 3 h after inoculation in the resistant as well as in the inducer treated seedlings, but the percentage of seedlings displaying HR varied with the treatment (Fig. 2.1). In resistant, 20% seedlings showed the presence of HR at 3 h.a.i. whereas in inducer treated, seedlings showed 18% and 15% (conidia and CPE) of HR, respectively at the same time of interval. At the end of 24 h.a.i., 84% of resistant, 78% and 73% of inducer treated seedlings (conidia and CPE), respectively displayed the HR responses, but the susceptible seedlings recorded poor HR expression of 21% (Fig. 2.2).

![Fig. 2.1: Time course study of hypersensitive reaction (HR) in pearl millet seedlings treated with conidial suspension (T 1) and CPE (T 2) of PenC- JSB9 upon pathogen inoculation. Vertical bars indicate standard error. Results are average of three independent experiments with four replicates of twenty five seedlings each.](image-url)
Chapter 2

Results

Histological Studies

Lignin deposition

Pearl millet seedlings treated with inducers of PenC- JSB9 and SDW were subjected for identification of lignin deposition in cell wall in response to inoculation with the downy mildew pathogen by differential staining. Lignification was observed as early as 3 h.a.i. in inducer treated and resistant inoculated seedlings, whereas there was no significant lignin deposition in susceptible inoculated seedlings up to 6 h.a.i. Rapidity of lignification in resistant, induced resistance and susceptible seedlings of pearl millet in both pathogen inoculated and uninoculated varied (Fig. 2.3). Microscopic observations of the stained epidermal peels showed lignin as reddish brown depositions along the cell wall and observations were recorded up to 24 h.a.i. (Fig. 2.4). Resistant inoculated seedlings offered maximum lignification when compared to inducer and susceptible inoculated seedlings. Maximum lignification of 86% was observed in resistant inoculated seedlings, followed by inducer treated which offered 74% and 68% in conidial and CPE treatment, respectively. In susceptible inoculated seedlings, no lignification was observed at the constitutive level, but showed a slight increase by the end of 24 h.a.i.
Fig. 2.3: Temporal pattern of the degree of lignifications in coleoptiles tissues of pearl millet seedlings treated with conidial suspension (T 1) and CPE (T 2) of PenC- JSB9 upon pathogen inoculation. Vertical bars indicate standard error. Results are average of three independent experiments with four replicates of twenty five seedlings each.
Fig. 2.4: Light microscopic pictures showing the deposition of lignin in the epidermal peelings of coleoptile region upon different modes of inducer treatment with PenC- JSB9 after 24 h.a.i. A-Resistant uninoculated; B- Resistant inoculated; C- Treated (conidia) uninoculated; D- Treated (conidia) inoculated; E- Treated (CPE) uninoculated; F- Treated (CPE) inoculated; G- Susceptible uninoculated; H- Susceptible inoculated. Arrows indicate lignin deposition. Bar = 3 µm
Callose deposition

The cells stained with aniline blue identified callose as bright greenish yellow fluorescence under Fluorescence microscope. Callose deposition in the cell wall of pearl millet seedlings induced with PenC- JSB9 with different mode in response to inoculation with *S. graminicola* was studied by differential staining. Rapidity of callose deposition in resistant, induced resistance and susceptible seedlings of pearl millet in both pathogen inoculated and uninoculated varied (Fig. 2.5). The induced resistant pearl millet seedlings (conidia and CPE treatment) showed maximum deposition upon inoculation with pathogen than in uninoculated seedlings (Fig. 2.6). There was about 4.87% difference in callose deposition in cells of pearl millet seedlings treated with conidia and CPE upon inoculation, but there was a marked difference when compared with susceptible inoculated seedlings at 24 h.a.i. The induced seedlings showed callose deposition as early as 3 h.a.i. and increased thereafter till 24 h.a.i. Maximum deposition of callose was observed in pearl millet seedlings resistant to *S. graminicola* compared to the plants susceptible to the disease. In early stages, there was no much difference in the deposition of callose but gradually increased after 6 h.a.i. till 18 h.a.i. and plateaued later.

![Fig. 2.5: Temporal pattern of the degree of callose deposition in coleoptile tissues of pearl millet seedlings treated with conidial suspension (T1) and CPE (T 2) of PenC- JSB9 upon pathogen inoculation. Vertical bars indicate standard error. Results are average of three independent experiments with four replicates of twenty five seedlings each.](image_url)
Fig. 2.6: Light microscopic pictures showing the deposition of callose in the epidermal peelings of coleoptile region upon different modes of inducer treatment with PenC- JSB9 after 24 h.a.i. A- Resistant uninoculated; B- Resistant inoculated; C- Treated (conidia) uninoculated; D- Treated (conidia) inoculated; E- Treated (CPE) uninoculated; F- Treated (CPE) inoculated; G- Susceptible uninoculated; H- Susceptible inoculated. Arrows indicate callose deposition. Bar = 3 µm
Biochemical studies

Estimation of Phenylalanine Ammonia Lyase (PAL) activity

The pearl millet seedlings treated with inducers (conidial suspension and CPE of PenC- JSB9) showed increased level of PAL activity at all time intervals of 0, 3, 6, 9, 12, 24, 48 and 72 h.a.i. when compared to control (Fig. 2.7). A progressive increase in PAL activity was observed from 0 h.a.i. and reached maximum at 24 h.a.i. irrespective of the treatments and pathogen infection. PAL activity decreased or remained constant beyond 24 h.a.i. in all the treatments. However, maximum PAL activity was observed in resistant challenge inoculated seedlings (345.2 U) followed by conidial suspension treated inoculated seedlings (318.2 U) and CPE treated inoculated seedlings (309.5 U). The susceptible treated, uninoculated seedlings showed PAL activity of 256.4 U and 238.8 U in conidial and CPE treatments, respectively. The observed difference in PAL activity between the untreated and elicitor treated seedlings was more than 2-fold and between susceptible and resistant was about 2.6 fold.

![Graph showing the temporal pattern of PAL activity](image-url)
Estimation of Peroxidase (POX) activity

POX activity was estimated in both inoculated and uninoculated seedlings raised from resistant, PenC- JSB9 inducer treated and susceptible cultivars. The POX profile at 0, 3, 6, 9, 12, 24, 48 and 72 h.a.i. is depicted in Fig. 2.8. The pearl millet seedlings raised from elicitor treated (conidial suspension and CPE) and challenge inoculated seeds showed higher level of POX activity of 67 U and 65.4 U at 48 h.a.i. compared to susceptible seedling which offered 42.2 U at same time interval. Thus, protected seedlings showed 35% increase in enzyme activity at 48 h.a.i. over the control and the increase in enzyme activity was maintained thereafter. There was no significant increase in POX activity in SDW treated pearl millet seedlings upon inoculation. POX activity in resistant seedlings was higher compared to induced resistant. However, POX activity in control seedlings remained lesser than the induced and resistant seedlings.

Fig. 2.8: Temporal pattern of accumulation of POX enzyme in pearl millet seedlings upon different modes of inducer treatment with PenC- JSB9. RU-Resistant uninoculated; RI- Resistant inoculated; ST1-U- Susceptible treated (conidial suspension) uninoculated; ST1-I Susceptible treated (conidial suspension) and inoculated; ST2-U- Susceptible treated (CPE) uninoculated; ST2-I- Susceptible treated (CPE) and inoculated; SU- Susceptible uninoculated; SI- Susceptible inoculated. Data of enzyme activity are means of three different experiments and bars indicate ± SE.
Estimation of β-1,3-Glucanase activity

Resistant and inducer treated pearl millet seedlings showed elevated levels of β-1,3-glucanase activity over susceptible seedlings. Maximum β-1,3-glucanase activity was observed at 12 h.a.i. in all the samples and thereafter remained constant or decreased. Maximum β-1,3-glucanase activity upon *S. graminicola* infection was observed in resistant seedlings of 1.4 μmol/ min/ mg protein followed by conidial suspension and CPE treatment of 1.20 and 1.05 μmol/ min/ mg protein, respectively at 12 h.a.i. The enzyme activity in uninoculated seedlings were 0.65 μmol/ min/ mg protein, while it decreased on pathogen inoculation (0.55 μmol/ min/ mg protein) at same time interval (Fig. 2.9).

![Fig. 2.9: Temporal pattern of accumulation of β-1, 3-Glucanase enzyme in pearl millet seedlings upon different modes of inducer treatment with PenC-JSB9. RU-Resistant uninoculated; RI- Resistant inoculated; ST1-U- Susceptible treated (conidial suspension) uninoculated; ST1-I Susceptible treated (conidial suspension) and inoculated; ST2-U- Susceptible treated (CPE) uninoculated; ST2-I- Susceptible treated (CPE) and inoculated; SU- Susceptible uninoculated; SI- Susceptible inoculated. Data of enzyme activity are means of three different experiments and bars indicate ± SE.](image-url)
Estimation of Chitinase activity

Chitinase activity was selected as a host defense response in order to verify the induced resistance which is involved in protection exerted by elicitor treatment. A differential response of chitinase activity was observed in all the samples treated with elicitors. Two-day-old seedlings on challenge inoculation showed steady increase of chitinase activity up to 24 h.a.i. and decreased at all other time points tested (Fig. 2.10). The elicitor induced significantly higher chitinase activity (5.6 and 5.31 n mol/min/mg protein in conidial and CPE treated, respectively) in inoculated seedlings than in susceptible inoculated seedlings (2.1 n mol/min/mg protein). The resistant and induced resistant seedlings upon challenge inoculation showed increased activity, while it decreased in susceptible inoculated.

Fig. 2.10: Temporal pattern of accumulation of chitinase enzyme in pearl millet seedlings upon different modes of inducer treatment with PenC-JSB9. RU-Resistant uninoculated; RI- Resistant inoculated; ST1-U- Susceptible treated (conidial suspension) uninoculated; ST1-I Susceptible treated (conidial suspension) and inoculated; ST2-U- Susceptible treated (CPE) uninoculated; ST2-I- Susceptible treated (CPE) and inoculated; SU-Susceptible uninoculated; SI- Susceptible inoculated. Data of enzyme activity are means of three different experiments and bars indicate ± SE.
Lipoxygenase activity (LOX)

The time course study in elicitor treated seedlings followed by pathogen inoculation recorded maximum LOX activity at 48 h.a.i. The pathogen inoculation in elicitor treated, resistant and control seedlings, the LOX activity was evident at 6 h.a.i. and reached maximum at 48 h.a.i. and maintained thereafter. An increase of 34% and 25% LOX activity was observed in conidial suspension and CPE treatment, respectively when compared to control. Similarly, in resistant inoculated pearl millet seedlings showed higher LOX activity than inducer treated inoculated seedlings. The control inoculated and uninoculated seedlings recorded 28.8 U and 23.2 U at 48 h.a.i., respectively (Fig. 2.11).

**Fig. 2.10. Temporal pattern of accumulation of LOX enzyme in pearl millet seedlings upon different modes of inducer treatment with PenC-JSB9.** RU-Resistant uninoculated; RI- Resistant inoculated; ST1-U- Susceptible treated (conidial suspension) uninoculated; ST1-I Susceptible treated (conidial suspension) and inoculated; ST2-U- Susceptible treated (CPE) uninoculated; ST2-I- Susceptible treated (CPE) and inoculated; SU-Susceptible uninoculated; SI- Susceptible inoculated. Data of enzyme activity are means of three different experiments and bars indicate ± SE.
DISCUSSION

Biomolecules like oligosaccharides, glycoproteins, glycopeptides and lipids are known to be involved in detection of pathogen and induction of defense responses in plants (Ebel et al., 1994; Boller, 1995; Hahn, 1996; Hindumathy, 2012). Induced resistance in plants is correlated with defense reaction markers which include both biochemical markers like involvement/induction of PR proteins and histological markers, such as HR, deposition of lignin and callose. Chemical substances secreted or present on the surface of the pathogen pave the way for its detection by plant. It was clear from Chapter 1 that conidial suspension and CPE of PenC- JSB9 were promising in protecting plants against downy mildew infection and improving growth and development of pearl millet compared to TriH- JSB25 and PhoS-32. Hence, in this chapter we have undertaken studies on effect of elicitor/inducer (conidial suspension and CPE) of PGPF-PenC- JSB9 on defense responses during pearl millet downy mildew host-pathogen interactions at morphological, histological and biochemical level.

Local defense is commonly associated with the HR and programmed cell death in plants (Heath, 2000). HR is visually observed as brown necrotic spots or streaks representing localized cell death and as resistance response in interaction between plants in general and to downy mildew oomycetes in particular (Dangl et al., 1996; Kamoun et al., 1999). There was a rapid expression of HR in the resistant seedlings while the susceptible seedlings showed HR at later hours after inoculation. The early HR response in PGPF treated seedlings, similar to that of resistant reflects the rapid response to the pathogen infection sensitized by elicitors. The difference in rapidity and degree of HR as a response to inoculation with S. graminicola observed could be due to difference in expression of associated resistance genes. Such variation depending on the interacting pathogen and plant genotype has been documented (Kamoun et al., 1999). HR like response was observed in suspension cell cultures of grapevine treated with an elicitor (cellulase, Onozuka R-10) isolated from T. viride (Zapata et al., 1994). HR as a defensive response and as a marker of resistance has been established earlier in pearl millet downy mildew interaction with various inducers like D. metel (Kumudini et al., 2001; Shivkumar et al., 2009), BTH (Geetha and Shetty, 2002), unsaturated fatty acids (Amruthesh et al., 2005) and PGPR (Niranjanraj et al., 2012).
Chapter 2 Discussion

Lignin deposition is known as one of the mechanisms of ISR (Hammaerschimidt and Kuc, 1982). Stadnik and Buchenauer (1999) hypothesized the presence of phenolic compounds during the process of lignification resulting in cell strengthening. Furthermore, lignin is known to be potentially toxic to the pathogen, and adversely prevents the pathogen entry. Lignin deposition in pearl millet seedlings induced by elicitors of PenC- JSB9 treatment was evidenced as an important host defense response against S. graminicola infection. Maximum lignification was observed at 24 h.a.i. in all the seedlings and lignification was noticed as early as 3 h.a.i. However, the intensity and rapidity of lignin deposition varied among the resistant, elicitor treated and susceptible seedlings. Koike et al. (2001) reported enhanced lignification in cucumber seedling hypocotyls treated with culture filtrates of PGPF, following challenge inoculation with C. orbiculare when compared to control.

Further, in the present study callose deposition has also been recorded in elicitor treated pearl millet seedlings. Callose, a β-1,3-glucan polysaccharide, is a product of a highly localized defense reaction which is deposited in wound plugs and papillae. It provides a mechanical barrier at the site of pathogen entry to restrict growth and spread of the pathogen and hence involved in host defense. Similar trend of callose deposition was noticed as in lignification and maximum callose deposition was observed in resistant seedlings. Callose deposition was observed more prominently along cell wall in elicitor-treated samples in response to S. graminicola infection. Callose deposition was reported in soybean- Phytophthora sojae system (Enkerli et al., 1997) and in rice seedlings treated with elicitor MoHrip1 isolated from M. oryzae (Chen et al., 2012).

The role of cell wall lignification and callose deposition enriched in proteins and autofluorescing phenolic compounds has been reported in French bean against Xanthomonas campestris (Brown et al., 1998) and barley against Blumeria graminis (Lyngkaer and Carver, 1999). Similar results have been obtained by Hindumathy et al. (2006) and Niranjan Raj et al. (2012) where an increase in lignin deposition was observed as time-gap increased in seedlings treated with spore cell wall components of A. niger and B. pumilus strain INR-7, respectively. Because the coleoptiles serve as an essential path for S. graminicola to spread systemically to upper tissues, a rapid and localized increase of lignin and callose in this region is supposed to contribute to
resistance. Increased level of lignin and callose deposition noticed in the plasma membrane is essential because it is found that *S. graminicola* downy mildew pathogen of pearl millet spreads systemically to upper tissues through these regions and increased deposition of lignin and callose is supposed to contribute to disease resistance (Sharada *et al.*, 1995).

Pathogenesis related (PR) proteins are induced in pathological situations in plants, (Bowles, 1990) produced via salicylic-dependent pathway and considered a part of the multiple defense systems of plants (Kombrink and Somssich, 1997). PAL, a key regulatory enzyme of phenylpropanoid metabolism, catalyzes the conversion of L-phenylalanine into of trans -cinnamic acid with the elimination of ammonia. *Trans*-cinnamic acid is a precursor of a wide range of phenolic metabolites, phenylpropanoids, regulatory signaling molecules and other compounds and it also activates secondary defense responses (Takahashi *et al.*, 2001). PAL has been demonstrated in metabolic activity of many higher plants and in 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 and intensively studied because of its key role in phenylpropanoid biosynthesis (Whetten and Sederoff, 1995). Geetha *et al.* (2005) suggested the involvement of PAL in resistance mechanism of pearl millet to *S. graminicola*. The temporal pattern of PAL activities in resistant, induced resistant and susceptible pearl millet cultivars showed significant induction after pathogen infection and at 24 h.a.i. the enzyme activity reached peak. Increased activity in all the test seedlings after pathogen inoculation and higher activity in resistant compared to induced resistant and susceptible cultivars indicates a possible role for PAL during pathogen infection and host resistance. PAL activity in conidial suspension treated seedlings was higher than CPE treated and control pearl millet seedlings. A multiple fold PAL activity was observed in resistant and induced resistant seedlings after challenge inoculation when compared to susceptible seedlings without infection. Similar results were obtained in pearl millet by Sudisha *et al.* (2011), wherein seed treatment with raw cow milk and amino acids enhanced PAL activity by 5 and 8 folds, respectively when compared to control. There was an early increase in PAL activity in rice upon infection with *P. oryzae* (Wang *et al.*, 2004) and in barley in response to fungal pathogens and elicitor treatments (Kervinen *et al.*, 1998). Our
Results are consistent with many earlier findings where PAL activity was higher in cultured cells of resistant bean in response to *C. lindenuthianum* infection and in maize and tobacco due to pathogen infection (Edwards *et al*., 1995; Perrone *et al*., 2000; Forlani, 2002).

POX as an enzyme with multifaceted activities acting in both biotic and abiotic stress responses, mostly synthesized in the chloroplasts (Gabara *et al*., 2003). Plant POX exists as isoenzymes with diverse expression profiles ranging from HR, lignification, cross-linking of phenolics, glycoproteins, and suberisation to phytoalexin production (Griesbach, 1981; Baysal *et al*., 2003). It catalyses the condensation of phenolics into lignin produced during pathogen infection, which acts as an antimicrobial compound. It is also involved in detoxification of reactive oxygen species (ROS) in plants during pathophysiological stress (Kawano, 2003). POX in several plant systems have been shown to be altered by stress, chemicals and infection (Herbette *et al*., 2003; Sasaki *et al*., 2004) in higher plants (Sasaki *et al*., 2007) and its spatio-temporal induction by pathogen (Kishore *et al*., 2005; Huang and Backhouse, 2005). Role of POX in all the above-mentioned processes has been proved in downy mildew of pearl millet (Shivakumar *et al*., 2003). POX activities in pearl millet, has shown to be associated with reduction in the rate of pathogen multiplication and spread (Sudisha *et al*., 2011; Nairanjan Raj *et al*., 2012). Maximum elicitation of POX was achieved at 48 h.a.i. with 35% increase over uninoculated control, which is comparable to 45% increase in spore cell wall components of *A. niger* treatment at 8 h.a.i. (Hindumathy *et al*., 2006). Similar comparable data have been achieved by oligosaccharides of *Trichoderma* spp. (Nandini *et al*., 2013) in pearl millet. Similar induction of enzyme activity found against *A. solani* and *P. infestans* in tomato plants treated with *T. harzianum* (Chowdappa *et al*., 2013). High POX activity has been associated with resistance in pepper to *P. capsici* (Alcazar *et al*., 1995) and in tomato against *F. oxysporum* f.sp. *lycopersici* and *A. alternata* (Hameed *et al*., 2010).

Plant chitinases are numerous and highly diverse, arranged in various classes and into endo- and exo-acting enzymes and are no doubt important in pathogenesis (Samac *et al*., 1990; Huynh *et al*., 1992; Wu *et al*., 1994; Berger *et al*., 1995; Ponath *et al*., 2000). Chitinases may be acidic or basic, vacuolar or cytoplasmic, constitutive or induced by various stresses including disease challenge. There is often a base level of chitinase activity in different species and increase after a stress event which can be
due to activation of different chitinase genes (Taylor et al., 1990; Mc Fadden et al., 2001). The enhanced protection by the chitinases could be due to direct inhibition of fungal growth or due to induction of plant defense responses by GlcNAc oligomers generated by their activity (Ishihara et al., 1988). Chitinase activity was maximum at 24 h.a.i. and increased upon infection in resistant and induced resistant while decreased in susceptible. The activity at all points was higher in resistant inoculated compared to induced resistant and susceptible. The observed changes in enzyme levels are a consequence of systemic induction by conidial suspension and CPE of PGPF, which have been noted in other studies as well (Yedidia et al., 2003; Shoresh et al., 2005; Marra et al., 2006; Shoresh et al., 2006; Alfano et al., 2007; Shoresh and Harman, 2008). Accumulation of chitinase against Phytophthora has also been reported in earlier studies (Meins and Ahl, 1989; Schroder et al., 1992; Siefert and Grossman, 1997). Infection of potato leaves by the late blight fungus P. infestans led to a strong increase in chitinase activity (Buchter et al., 1997). Several Trichoderma strains induce the ISR pathway (Djonovic et al., 2007; Shoresh et al., 2005) while others are suspected to induce the SAR pathway (Brunner et al., 2005). These data suggest that different pathways of induced resistance may be activated by the presence of different strains. It appears to be a very good system to examine the total potential of plants to express induced resistance and changes in chitinolytic enzymes can provide good markers for these differential responses (Samac et al., 1990; Huynh et al., 1992; Wu et al., 1994; Berger et al., 1995; Ponath et al., 2000).

Glucanses are constitutively present in different organs and tissues of higher plants and are regulated by normal developmental processes, ethylene and other plant hormones. β-1,3-Glucanases are also induced in plants after pathogen attack and exposure to various biotic and abiotic elicitors, and they usually act in synergy with chitinases (Mauch et al., 1988; Simmons, 1994). The results of the present study substantiated that constitutive glucanses is associated with resistance reaction in pearl millet downy mildew interaction as reported in various plants (Reuveni and Karchi, 1987; Reuveni et al., 1991; Ahl Goy et al., 1992; Mozzetti et al., 1995). In our study, higher activity of β-1,3-glucanase detected in resistant seeds was also found in induced resistant seedlings. Increase in enzyme activity in the resistant cultivars after inoculation and the activity reaching a peak at 24 h.a.i. suggested a role for this enzyme in the expression of effective resistance by pearl millet seedlings to S.
graminicola invasion since in downy mildew disease, maximum infection of the host by the fungus is achieved within 24 h.a.i. and comparable with the study of Subramanya et al. (1983). Cell wall glucan elicitor extracted from the potential biocontrol strain T. harzianum Th10 upon seedling treatment recorded an increased glucanase activity in treated seedlings when compared with control seedlings (Sriram et al., 2009). Similar increases in activity and accumulation of glucanase and chitinase enzymes in incompatible interactions of maize, pepper, barley and wheat with pathogenic fungi have been reported (Cordero et al., 1994; Kim and Hwang, 1994; Roulin et al., 1997; Caruso et al., 1999), suggesting a role for these enzymes in determining resistance against fungal pathogens.

LOX has several functions in plants in response to wounding, stress and pathogen attack (Kolomiets et al., 2001; Gao et al., 2008). LOX is known to have a central role in the regulation of the biosynthesis of several secondary metabolites, including terpenoids, phenylpropanoids and antioxidants and usually the constitutive activation of these metabolites are occurring around a few days after germination (Avanci et al., 2010). In this study, we reported that enhancement of LOX enzyme during pearl millet- downy mildew disease interaction. The constitutive activity of LOX was seen in all the cultivars. But there was marked difference in the enzyme activity in resistant, induced resistant and susceptible seedlings. In the current studies, inoculated resistant and PGPF treated seedlings, the LOX activity was evident at 6 h.a.i. and reached maximum at 48 h.a.i. Increased LOX activity was observed in inoculated seedlings treated with conidial suspension and CPE at 48 h.a.i. Increased LOX activity was also noticed in untreated inoculated samples. Further, the distilled water-treated control of both resistant and susceptible pearl millet seedlings exhibited a constant increase in LOX activity, even without elicitor-treatment or pathogen inoculation indicating the constitutive level of the enzyme. An increase in LOX activity in response to infection has been reported in many host-pathogen system and correlated with plant resistance against pathogens (Kolomiets et al., 2001, Porta et al., 2008). In resistant tobacco plants inoculated with P. parasitica f.sp. nicotianae LOX activity was maximum at 3 days, whereas in the susceptible plants the activity reached to maximum one day later (Veronesi et al., 1996). This study clearly supports the LOX is involved during development of downy mildew disease resistance in pearl millet and can be used as a marker for screening disease resistance. Thus, resistant,
induced resistant and susceptible seedlings showed differential accumulation of LOX activity in response to the pathogen infection. Increased LOX activity has been observed in several incompatible host-pathogen combinations (Dangl and Jones, 2001; Chandrashekar et al., 2007). Similar results were observed in pearl millet on infection with the pathogen. The LOX activity increased in the resistant seedlings compared to respective uninoculated controls. In pigeon pea infected with *F. udum*, the LOX activity increased 10 fold in the resistant cultivar over a period of 4 days following infection (Uma et al., 2000). Other examples include, the induction of LOX activity up to 7 fold in tobacco leaves over a period of 11 days following infection with *Erysiphe cichoracearum* (Lupu et al., 1980).

An elicitor, cerebrosides isolated and partially purified from *F. oxysporum* f.sp. *lycopersici* was found to stimulate PAL, POX and LOX activity when applied to chilli plants by spray treatment which sustained throughout the experimental period with or without modifications (Naveen et al., 2013). The above findings suggest the role for these defense-related molecules in the expression of induced resistance by oligosaccharide elicitor against *S. graminicola* invasion in PM (Nandini et al., 2013). In previous evidence, a fungal protein elicitor PebC from *A. tenuissima* could induce (Li et al., 2005) PAL and POX against *B. cinerea* in tomato. Protein elicitor from *S. graminicola* induced activation of PAL and POX activity (Sharthchandra et al., 2006) and homologous proteins like INF1, NPP1 (Baillieul et al., 2003), cryptogein, PeF (Orsomando et al., 2003), PB90 (Wang et al., 2003), megaspermin and quercinin (Koehl et al., 2003) with the similar biological activity have been found in other *Phytophthora* spp., an oomycete plant pathogen.

From the current study, it is understood that the elevated levels of defense-related enzymes in CPE and conidial suspension treated pearl millet seedlings and their further increase after *S. graminicola* infection indicates that seed treatment formed an incompatible setting for infection, proliferation and sporulation by *S. graminicola* leading to disease suppression. Hence, the defense responses exhibited by host upon elicitation by PGPF can be used as a biochemical marker for downy mildew disease resistance. Further, the defense responses at the molecular level are studied through mRNA transcript accumulation upon PGPF elicitor treatment in the forthcoming chapter.