



# Synchronised regulation of disease resistance in primed finger millet plants against the blast disease



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## ABSTRACT

Plants, being sessile, are exposed to an array of abiotic and biotic stresses. To adapt towards the changing environments, plants have evolved mechanisms that help in perceiving stress signals wherein phytohormones play a critical role. They have the ability to network enabling them to mediate defense responses. These endogenous signals, functioning at low doses are a part of all the developmental stages of the plant. Phytohormones possess specific functions as they interact with each other positively or negatively through cross-talks. In the present study, variations in the amount of phytohormones produced during biotic stress caused due to *Magnaporthe grisea* infection was studied through targeted metabolomics in both primed and control finger millet plants. Histochemical studies revealed callose deposition at the site of pathogen entry in the primed plants indicating its role during plant defense. The knowledge on the genetic makeup during infection was obtained by quantification of MAP kinase kinases 1 and 2 (*MKK1/2*) and lipoxygenase (*LOX*) genes, wherein the expression levels were high in the primed plants at 6 hours post-inoculation (hpi) compared to mock-control. Studies indicate the pivotal role of mitogen-activated protein kinase (MAPK or MAP kinases) during defense signalling. It is the first report to be studied on MAPK role in finger millet-blast disease response. Temporal accumulation of LOX enzyme along with its activity was also investigated due to its significant role during jasmonate synthesis in the plant cells. Results indicated its highest activity at 12 hpi. This is the first report on the variation in phytohormone levels in finger millet - *M. grisea* pathosystem upon priming which were substantiated through salicylic acid (SA) pathway.

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## 1. Introduction

Plant induced defense responses depend on the nature of phytopathogens and accumulation of host nutrition [1,2]. In general physiological changes and natural structural barriers present in the host plant responds quickly upon pathogen attack [3–6]. The essential metabolites for cell proliferation and growth are the primary metabolites, produced during the process of growth and maintain the fundamental metabolic process like

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photosynthesis and respiration [7]. Thus, the key primary metabolites with energy sources viz., sugars, amino acids, tricarboxylic acids, organic acids, nucleic acids and polysaccharides are considered to play a vital role in metabolic process [8–10]. In general, the photosynthetic machinery and primary metabolism are suppressed during pathogen encounter due to the energy consumption by the host towards defense response against the pathogen. However, the recent reports [10] describe that the primary metabolism also helps in confronting the pathogen attack.

Plant immunity is regulated by the phytohormones through antagonistic or /and synergistic interactions contingent to the environmental status. The regulatory mechanism is triggered by the plant hormones through the pathogen-specific elicitors by the activation of downstream regulatory mechanisms and R genes. Hence, the sparking/ elicitors that trigger the defense system include salicylic acid (SA) and jasmonic acid (JA) [2,10–12]. SA is a major hormone in the plant immune system which is crucial to establish local and systemic immune response against a wide range of pathogens [13]. The biotrophic response is regulated by SA, while ET and methyl jasmonates play a significant role in controlling the necrotrophs [14,15]. Gibberellic acid, abscisic acid and auxins play a role in plant growth and proliferation [16].

In this section, the secondary metabolite (phytohormone) variations during the biotic stress posed by *Magnaporthe grisea* in primed and control plants was deduced by targeted metabolomics approach. The genetic makeup during pathogen encounter was also studied by studying the gene expression of the MAP kinase kinases 1 and 2 (*MKK1/2*) and lipoxygenase (*LOX*) genes were quantified with the aid of real-time quantitative polymerase chain reaction (RT-qPCR). The *LOX* enzyme activity was also studied in the temporal pattern as the reaction catalysed by this enzyme is decisive in JA synthesis in the plant cells.

## 2. Materials and Methods

### 2.1. Plant Material, Treatments and Sample Collection

Blast disease susceptible finger millet variety (Indaf 9) was collected from MAS Lab, GKVK, Bengaluru. The pathogen *Magnaporthe grisea* (MTCC-1477) was procured from IMTECH, India. The culture was maintained on oat meal agar until further use. The beneficial bacteria (inducers) were selected from the previous study by Patil et al. [17]. The two *Pseudomonas aeruginosa* (JUPC113: KX010601) and (JUPW121: KX010602) isolates were used in the present study.

Finger millet seeds were disinfected and pre-sterilized with sodium hypochlorite solution (0.2 %) for 5 min and rinsed three times with sterile distilled water (SDW). The sterilized seeds were then primed with the corresponding isolates as per Patil et al. [17].

Then these were grown under green house conditions (temp:  $27 \pm 3^\circ\text{C}$ ; relative humidity (RH):  $65 \pm 5\%$ ), with three replications for each of the treatments control (un-primed), challenged control (unprimed, pathogen challenged), JUPC113 and JUPW121 (primed). Forty-day old plants were challenge inoculated with the spore suspension of ( $\sim 2 \times 10^6 \text{ ml}^{-1}$ ) of *M. grisea* by foliar spray method [17].

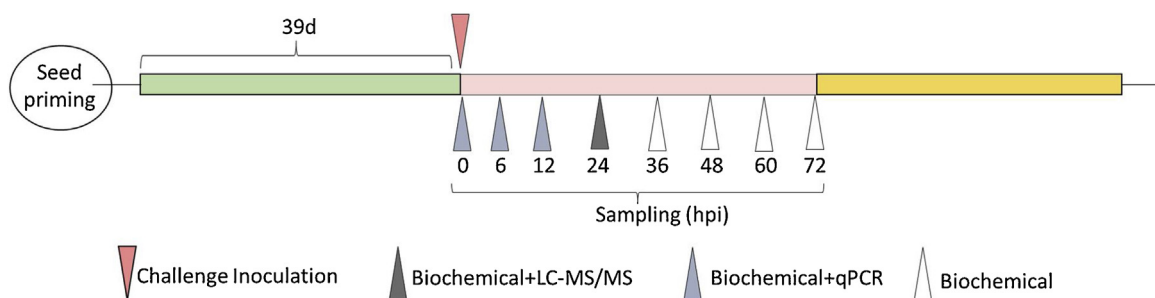
Leaf sampling was done at 0, 6, 12, 24, 36, 48, 60 and 72 hours post-inoculation (hpi) for the biochemical studies. The leaves were also sampled at 72 hpi for the metabolomic studies of different hormones (Fig. 1). The samples were frozen at  $-80^\circ\text{C}$  until use for better storage. Leaves collected at 0, 6 and 12 hpi were frozen by dropping in liquid nitrogen after collection within a fraction of minute for RNA isolation. This ensures the containment and integrity of the RNA for real-time quantitative polymerase chain reaction (RT-qPCR) studies.

### 2.2. Histochemical localization of defense molecules

For histochemical studies, different set of seedlings were used for all treatments. Coleoptiles of seedlings were excised after 72 hpi and fixed in acetic acid : ethanol (1:3; v/v). The samples were macerated with 3 % sodium hydroxide (w/v) for 1 h at  $65 \pm 2^\circ\text{C}$ . The samples were thoroughly washed with SDW and further used to stain the various host defense compounds. The peelings were stained with water-soluble 1 % aniline blue for 1 h and mounted with glycerol as per the method by Shetty et al. [18]. The callose deposited was visualized under a fluorescence microscope (Labomed, LX400, India) ( $\lambda_{350-410 \text{ nm}}$ ) at 100x magnification. Fresh leaves excised from plants after 72 hpi, were boiled in 95 % ethanol (v/v) until chlorophyll was decolourised. Further, they were immersed in 1 % (w/v) sodium dodecyl sulphate (SDS; Qualigens) for three days at  $80^\circ\text{C}$ , in order to sequester the soluble proteins. After incubation, the samples were stained with 0.1 % (w/v) coomassie blue prepared in ethanol : acetic acid (4:1; v/v) and washed with the same solution and mounted with distilled water. The protein cross-linking on the cell wall was visualized under various magnifications of the fluorescence microscope [19].

### 2.3. Targeted metabolomics on phytohormone levels

The phytohormone extraction and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) analysis was carried out as per Pan et al. [20] and Jogaiah et al. [2] with minor modifications. Briefly, 3.0 g of leaf sample was completely homogenised with a mixture of 1-propanol,  $\text{H}_2\text{O}$  and concentrated HCl (2:1:0.002; v/v/v), and mixed for 30 min at  $4^\circ\text{C}$  for overnight. Ten ml of Dichloromethane was added to the homogenate, re-shaken for 30 min and then centrifuged at  $12,000 \times g$  for 10 min. The bottom



**Fig. 1.** Scheme of leaf sampling at different time points. Lipoxygenase (*LOX*) activity was measured at all time points, hormonal studies was done at 24 hours post-inoculation (hpi) and gene expression studies was with 0, 6 and 12 hpi.

layer was mixed with 5 ml of sodium sulphate and evaporated by the evaporating chamber. The dried sample was dissolved in 80 % methanol and passed through the C18 solid-phase extraction (SPE) cartridges. The SPE process involves the preconditioning, adsorption and elution steps. Finally, the obtained elute (5 mL) was evaporated to dryness and dissolved in a mixture of 500  $\mu$ L methanol and 0.05 % formic acid (1:1; v/v). The solution was filtered using a nylon filter paper pore size (0.22  $\mu$ m size) and injected into LC-MS for further analysis.

The LC-MS/MS composed of an initial gradient with the mobile phase of solvent A as water, acetonitrile and acetic acid (95: 5: 0.05; v/v/v) and solvent B as acetonitrile, water and acetic acid (95: 5: 0.05; v/v/v). Then 85 % of solvent A and 15 % of solvent B was mixed and kept for 1 min, at 12<sup>th</sup> minute the gradient mixture changed to 15 % of A and 85 % of B and kept for 1 min, followed by linear gradient mixture containing 85 % of A and 15 % of B at 14<sup>th</sup> minute for 0.5 min. The system was finally returned to initial conditions at 15<sup>th</sup> minute and equilibrated for 1 min before the next injection. The flow rate was maintained at 0.2 mL/min. The analytical column of 2.1 x 50 mm UPLC BEH-C18 column (Waters, USA) with 1.7  $\mu$ m particles, protected by a vanguard BEH C-18 with 1.7  $\mu$ m was used for the study. The temperature was maintained at 25 °C. The elution was monitored using a TQD-MS/MS (Waters, USA) system, optimized for the hormone analysis.

#### 2.4. Gene expression studies

Total RNA was isolated using the plant RNA kit from Qiagen (74904) according to the manufacturer's instructions. Isolated RNA was resuspended in nuclease-free water and the integrity was analysed with 1 % agarose denaturing gel. The concentration of the total RNA content was determined using a nanodrop (Denovix DS-11, USA). First cDNA synthesis was carried out as per the instructions using the PrimeScript RT-PCR Kit from TaKaRa (Conalab, Barcelona, Spain) with the 1  $\mu$ g of total RNA concentration.

The real-time qRT-PCR was carried out using the Corbett thermocycler and analysed as per the manufacturer's instructions. The cDNA samples were diluted prior to the PCR setup with nuclease-free water. Reactions were carried out using the SYBR Premix Ex Taq kit from TaKaRa (Conalab), instructions followed on Corbett, Rotar Gene 6000 series. For the relative gene expression, actin was used as endogenous control and the gene-specific primer for MAP kinase kinases 1 and 2 (*MKK1/2*) and lipoxygenase (*LOX*) were used as listed in (Table 1). The PCR conditions were 94 °C for 30 s, 53 °C for 30 s and extension for 30 s at 72 °C with 40 cycles. The PCR conditions were optimized after checking the primer efficiency at different concentrations and temperatures in our laboratory. The standard curve was generated using the control samples, and the relative gene expression was expressed as fold change by  $2^{-\Delta\Delta Ct}$  method [21].

#### 2.5. Enzymatic activity of Lipoxygenase (LOX)

The leaves were ground in the freeze mortar and pestle using liquid nitrogen. To the powdered samples 0.2 M sodium

phosphate buffer (pH 6.5) was added and the LOX activity was measured as per Borthakur et al. [22]. The substrate, linoleic acid was prepared according to the method of Axelord et al. [23]. Briefly, 70  $\mu$ L of linoleic acid and an equal amount of Tween-20 with 3.0 mL of distilled water. The solution was cleared by adding 2 N NaOH, and final volume was made upto 25 mL with distilled water. The reaction mixture includes 2.7 mL of sodium phosphate buffer (0.2 M, pH 6.5  $\pm$  0.02) and 0.3 mL of the linoleic acid substrate. The absorbance was read at 234 nm after the addition of 2 mL of enzyme extract, and the activity was expressed as absorbance at 234  $\text{min}^{-1} \text{mg}^{-1}$  protein.

#### 2.6. Statistical Analyses

The experimental design was randomized throughout the study, consisting of three replications in greenhouse studies. Graph Pad Prism 8.2.0 version was used to generate graphs, and data were subjected to two-way ANOVA. Significance between the primed and control plants was obtained using Tukey's HSD test at  $p \leq 0.05$ . The clustering of heatmap and the Pearson correlation among treatments was examined using "corrplot" in R version 4.3.2 ([www.r-project.org](http://www.r-project.org)).

### 3. Results and Discussion

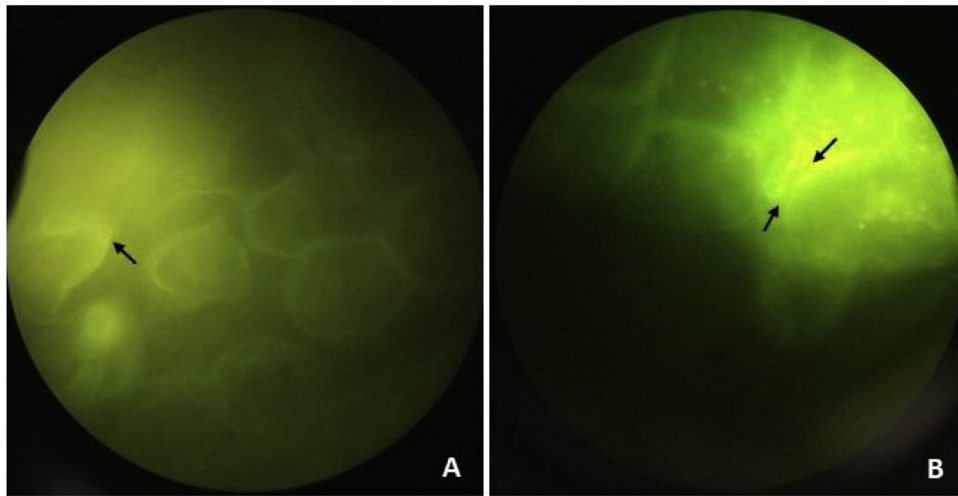
Plant-plant growth-promoting rhizobacteria (PGPR) interaction can induce physiological and biochemical changes in plants resulting in disease resistance [24]. Various studies [25–28] have shown that biological stress induces the production of defense-related antioxidant enzymes, which are further augmented by PGPR-mediated induction of disease resistance [29]. The augmented disease resistance elicits cell wall strengthening by limiting the pathogen progression. The major mechanical barriers posed by the host are lignification, callose deposition and crosslinking of proteins in the cell wall [30]. Thus in this study, the cell wall strengthening compounds deposited like callose and cross-linking proteins accumulated were studied histochemically. The phytohormonal variations incurred during induction of resistance and other signaling cascades by MAP kinase and LOX genes were studied quantitatively.

#### 3.1. Histochemical localization of defense molecules

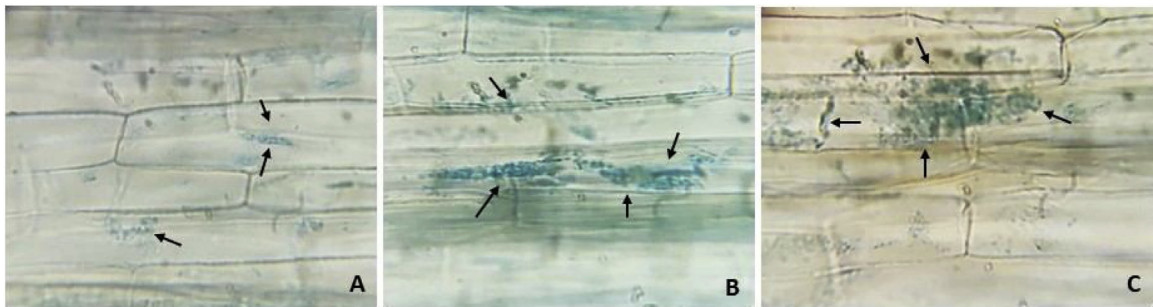
The present study revealed the deposition of callose in the site of pathogen entry in primed finger millet plants which was highly significant when compared to mock-control plants (Fig. 2). Thus this study emphasized the role of cell wall appositions during induction of disease resistance. The study also focussed on the deposition of callose, which can decipher the direct effect of antibiosis factors from the host and the inducer on the pathogen. The plant cells were stained for cross-linking proteins after pathogen challenge at 72 hours post-inoculation (hpi). The blue spots on the cell-walls (Fig. 3) are the cell-wall proteins stained by Coomassie blue which were not

**Table 1**  
Primer sequences used for the quantitative Real Time (qRT) -PCR studies

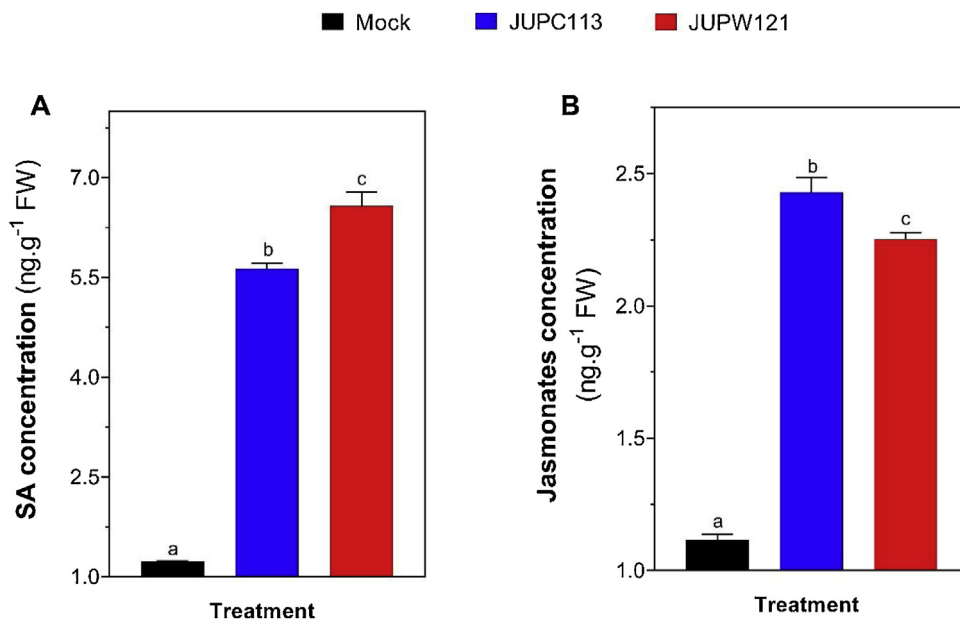
Primer	Forward (5'-3')	Reverse (5'-3')	Reference
<i>Actin</i>	GCCCTCCTCCTCCTCCTC	GATTATGGAGCGGGTGATGC	Kotapati et al. [44]
MAP kinase kinases 1 and 2 ( <i>MKK1/2</i> )	CAGAAGGAGAAGTTGGATAAGCT	GCAGGAAGGTGGTGGCTGATC	Li et al. [45]
<i>Lipoxygenase (LOX)</i>	CAGGCG TGGTGAAGGAG	GGACATCACGCCGAGTC	Kotapati et al. [44]



**Fig. 2.** Callose deposition at the cell wall region upon challenge inoculation in (A) mock-control and (B) primed leaves after 72 hours post-inoculation (hpi). Arrows (†) indicate the callose deposited on the cell walls.

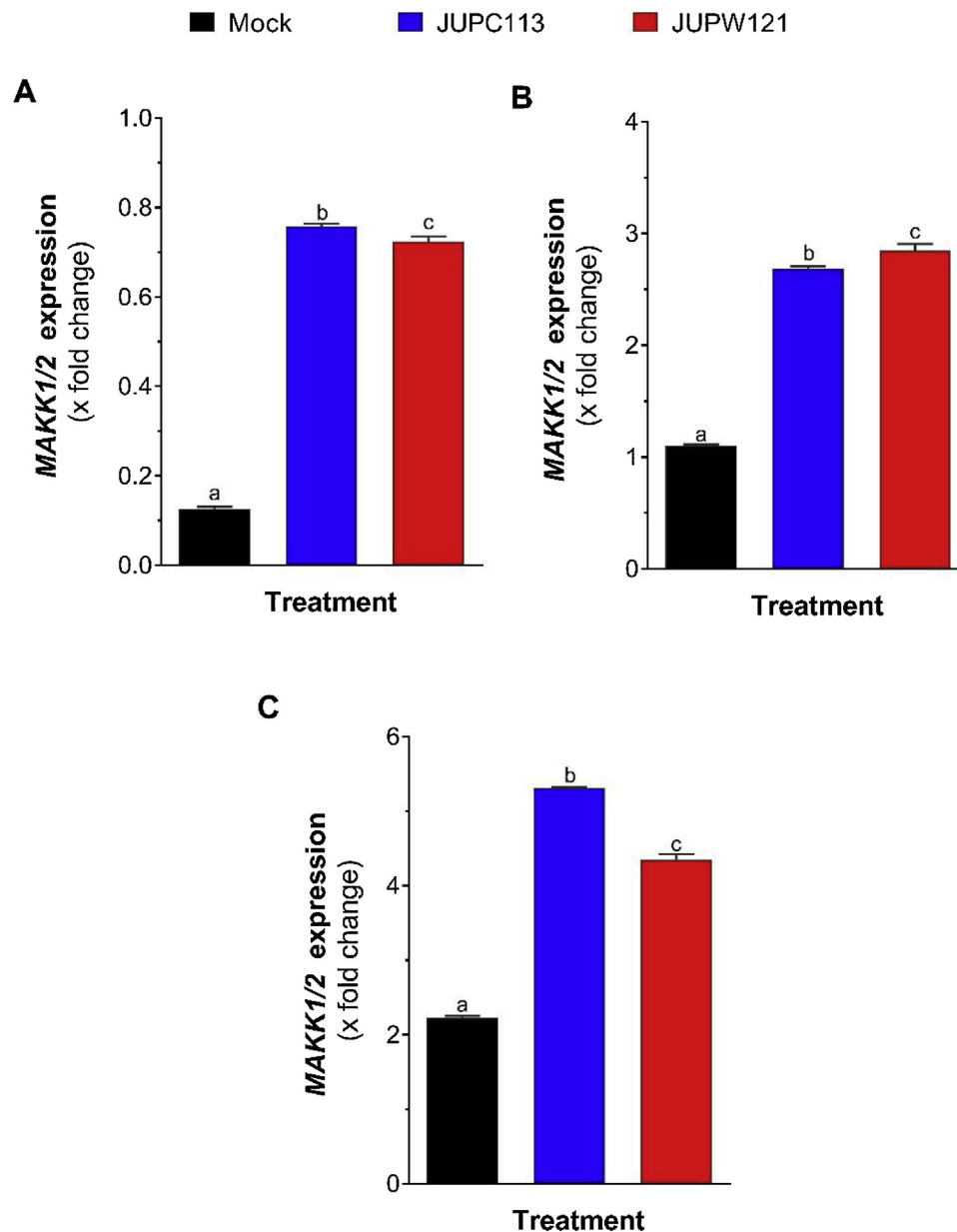


**Fig. 3.** Cross-linking proteins in the cell walls of (A) mock-control; (B) JUC113-primed and (C) JUPW121-primed after 72 hours post-inoculation (hpi). Arrows (†) indicate deposition of cross-linking proteins on the cell walls.



**Fig. 4.** Concentrations of phytohormones (A) salicylic acid (SA) and (B) jasmonates in mock-control and primed (JUC113 and JUPW121) plants after 72 hours post-inoculation (hpi).

Vertical bar represents mean  $\pm$  standard error (SE) ( $n = 3$ ) at  $p \leq 0.0001$  using Tukey's HSD test. Different letters (a, b, c) indicate the significant differences between the treatments.



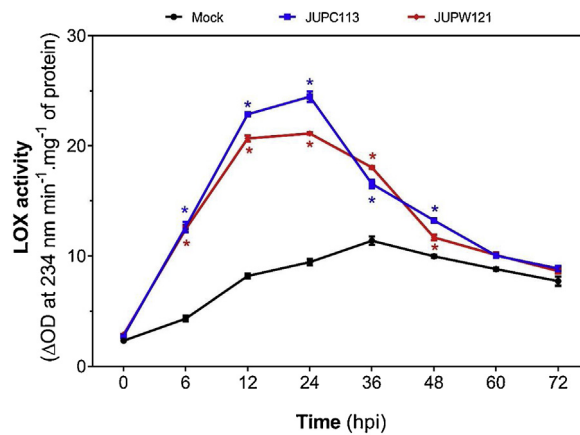
**Fig. 5.** Time dependent MAP kinase kinases 1 and 2 (*MKK1/2*) gene expression in mock-control and primed (JUPC113 and JUPW121) plants at (A) 0; (B) 6 and (C) 12 hours post-inoculation (hpi).

Vertical bar represents Mean  $\pm$  standard error (SE) (n = 3) at  $p < 0.0001$  using Tukey's HSD test. Different letters (a, b, c) indicate the significant differences between the treatments.

extracted out from the SDS. Thus, these proteins are efficiently required for retaining the wall proteins and cell structure, without damaging the cell components. These proteins include glycine-rich molecules involved in plant defense [31]. The cross-linking proteins also play a major role in the formation of wall apposition and hinder pathogen progression. Hence altogether these visual appositions against the pathogen is an important aspect for induction of resistance, by the signalling cascade of reactions from the preliminary hypersensitive reaction (HR) and lower levels of oxidative stress at the early hours of pathogen infestation.

### 3.2. Targeted metabolomics on phytohormone levels

Plant growth and the stress response is mediated by the secondary metabolites called phytohormones, which maintain the plant homeostasis to grow and sustain [32,33]. Therefore in this study, the phytohormones salicylic acid (SA), jasmonic acid (JA) known for their disease regulatory networks were studied through targeted metabolomics using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). The study evidenced the increase in levels of both the hormones in primed plants compared to the mock-control plants (Fig. 4).



**Fig. 6.** Time course activity of lipoxygenase (LOX) in mock-control and primed (JUPC113 and JUPW121) plants.

Vertical bar represents mean  $\pm$  standard error (SE) ( $n = 3$ ) at  $p \leq 0.0001$  using Tukey's HSD test. Stars indicate the significant differences between the treatments at different time interval.

The plants primed with JUPW121 showed the highest amount of SA which is highly significant compared to the mock control plants at  $p$  value  $\leq 0.05$ . JA levels were high in JUPC113-primed plants followed by JUPW121-primed and mock-control plants. Therefore, these studies demonstrated the role of SA-mediated defense response being imposed when primed with *P. aeruginosa* isolates. This is the first report on the study of variation in phytohormone levels in control and primed plants in finger millet - *Magnaporthe grisea* pathosystem which were substantiated through SA pathway.

### 3.3. Role of MAP kinase in plant defense

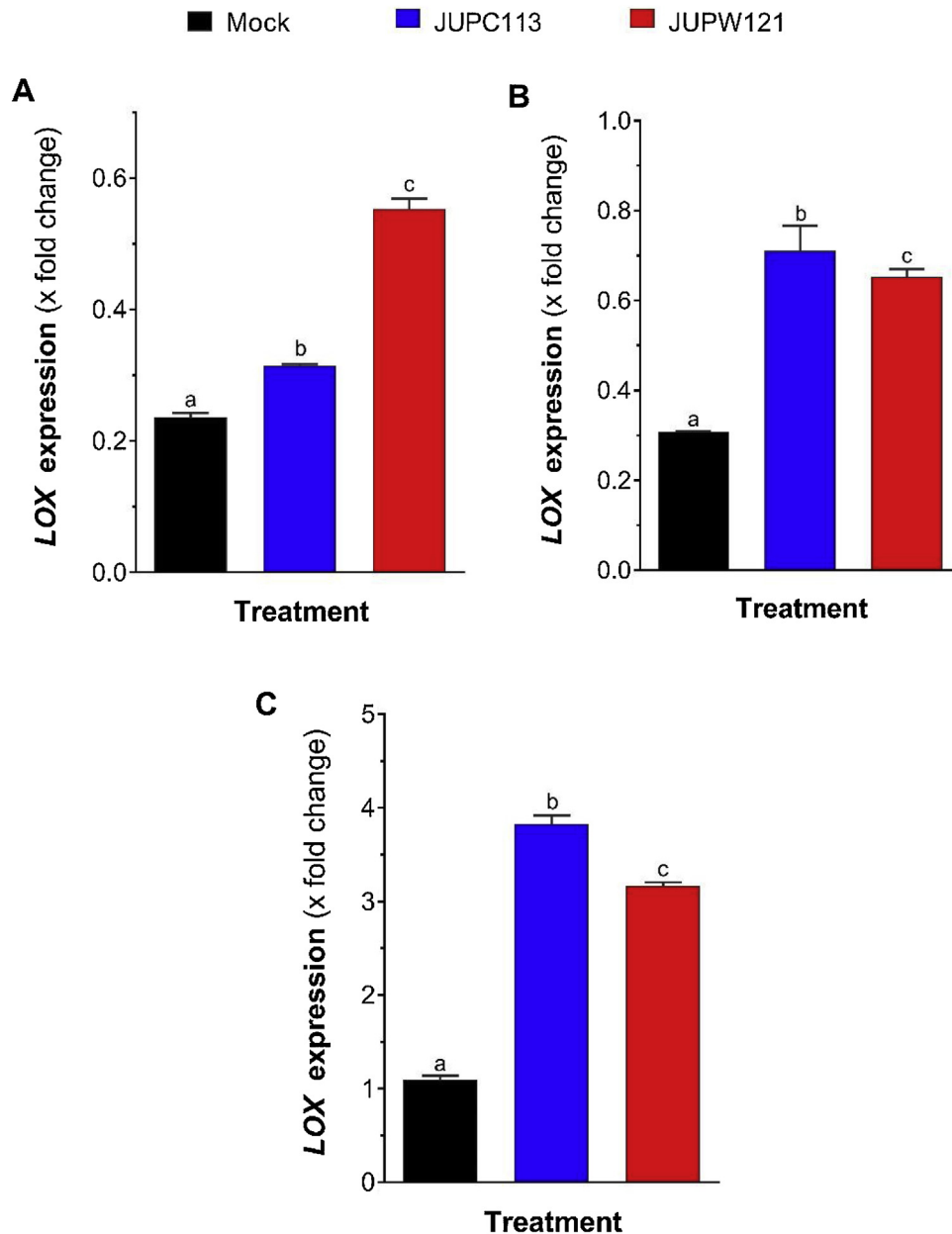
Mitogen-activated protein kinase (MAPK or MAP kinases) are the major regulators of any stress response in plants at an early period. They further signal the networked mechanism of disease resistance and pathogen regulation [34]. They are activated upon redox signal, and express through HR by Programmed cell death and in the host cells [35]. Therefore, to witness the regulation of *M. grisea* infection in finger millet plants, gene expression of MAP kinase kinases 1 and 2 (*MKK1/2*) was performed at 0, 6 and 12 hpi in mock and primed plants. The expression levels were high in both the primed plants at 6 hpi compared to mock-control (Fig. 5). The maximum fold change was observed at 12 hpi in JUPC113-primed plants ( $\sim 5$  fold) and followed by JUPW121 ( $\sim 4$  fold). Thus from this study, it was evident that MAPK plays a pivotal role in defense network signalling. It is the first report to be studied on MAPK role in finger millet-blast disease response. Studies revealed the presence of MAP3Ks (74), MAPKKs (9) and MAPKs (19) in maize plants through functional genomic studies [36]. Hence it was revealed that these MKK1 played a major role in pathogen defense response in both biotrophic and necrotrophic system where it played a differential function in necrotrophic versus biotrophic pathogen defense responses. Immunoblot studies by Melvin et al. [37] observed differential expression of MAPK related during the compatible and incompatible interactions between pearl millet and *Sclerospora graminicola*, a downy mildew pathogen. Hence, protein phosphorylation and dephosphorylation activates the defense alteration through MAPK cascade of signalling from external stimuli to the internal defense response.

### 3.4. Lipoxygenase (LOX)

LOX activity was found to be higher in primed plants with highest being in JUPC113-primed plants followed by JUPW121-primed and mock-control plants. The highest activity was observed at 24 hpi in JUPC113-primed plants. Previous studies using chemical elicitors reported an increased expression of LOX mRNA in pearl millet [38] which showed a maximum increase of LOX. Likewise, in the present study, an elevated LOX activity was observed at 24 hpi (Fig. 6) which was supported by a reduced blast disease incidence in finger millet. In addition, LOX gene expression was also found to be high in primed plants at 12 and 24 hpi in JUPC113-primed plants followed by JUPW121-primed (Fig. 7). The fold change was high at 12 hpi compared to 6 hpi in all treatments. Its expression also has been studied by Jacob et al. [39] in ragi - *M. grisea* infection.

Linoleic acid used for nutraceutical purposes, is a biologically beneficial functional lipid [40]. The biocidal effect of linoleic acid against gram-positive bacteria was reported by Dilika et al. [41]. Root treatment with linoleic acid significantly decreased the soft rot disease in tobacco at higher concentrations but was toxic to tobacco plants. Studies by Sumayo et al. [42] suggests that this can be more effective in eliciting ISR at lower concentrations, which also inhibited mycelial growth of *Alternaria solani*, *Fusarium oxysporum* f. sp. *Lycopersici* and *F. oxysporum* f. sp. *Cucumerinum* and exhibited an antifungal activity against *Crinipellis pernicioso*. Although it is reported that LOX is a principal player during induction of disease resistance, studies using microbial elicitors on LOX variation in plants are scarce (Enebe and Babalola [43]). Henceforth, the present study is important in the aspect of LOX-elicited-ISR in finger millet against blast disease. Though the LOX level was high in primed plants, both at gene and protein levels, it was not effective enough to synthesize JA due to compartmentalization. Hence, it can be plausible that LOX regulates other secondary metabolite and induce defense response against blast pathogen in finger millet plants.

Taken together, the results of phytohormone, gene expression and defense enzymes showed highly significant ( $p \leq 0.05$ ) interaction within treatments and between different time points. At 0 hpi, both treatments (JUPC113 and JUPW121) did not show any

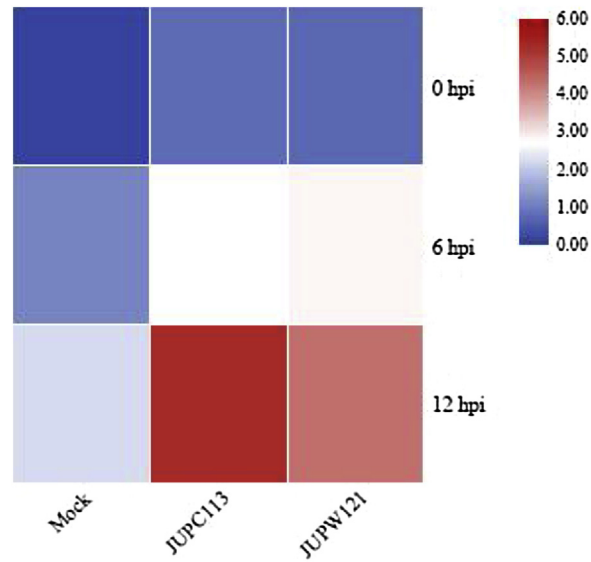


**Fig. 7.** Time dependent lipoxygenase (*LOX*) gene expression in mock-control and primed (JUPC113 and JUPW121) plants at (A) 0; (B) 6 and (C) 12 hours post-inoculation (hpi). Vertical bar represents Mean  $\pm$  standard error (SE) ( $n = 3$ ) at  $p \leq 0.0001$  using Tukey's HSD test. Different letters (a, b, c) indicate the significant differences between the treatments.

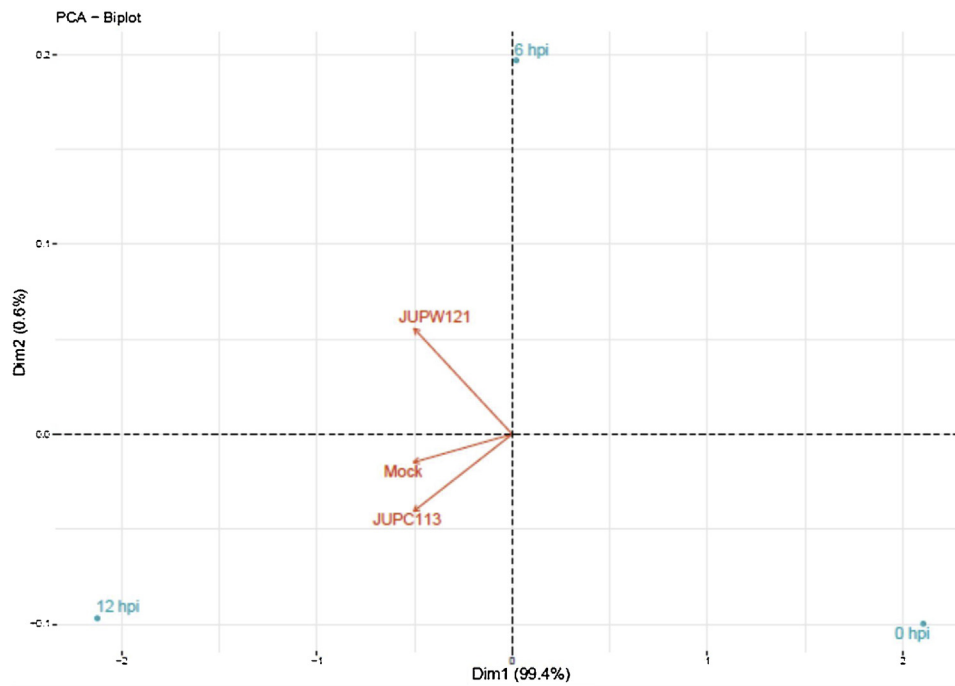
significant variation as compared to control (Fig. 8). However, JUPC113 showed significantly higher value (5.3), followed by JUPW121 (4.32) at 12 hpi as compared to control. Principal component analysis (PCA) was performed to check the variability within the treatment with respect to different time points. PCA result showed a total of 99.35% inertia in the dim1, which indicates the maximum variability within different treatments. All the vectors showed positive loadings in both axes (dim1 and dim2), while higher positive loading was recorded in MOCK (33.52), followed by JUPC113 (33.33) and JUPW121 (33.14) (Fig. 9). Overall, 0 hpi (49.51) and 12 hpi (50.48) contributed more in the dim1 as compared to 6 hpi (0.004). In addition, correlation analysis (CA) also depicted positive correlation between all treatments with

respect to increased time interval. All the treatments showed high positive correlation ( $R^2 = 0.98$ ) during different time intervals (Fig. 10).

The current study evidently suggests the role of key signalling plant hormones in induction of resistance, thereby enhancing the inducible protein synthesis and restricting the pathogen entry. Thus the signal cascade involves the various factors to direct the cell to attain the resistance power against the intruder, which needs to be further studied in with certain specific networks of inducible proteins. Nevertheless, the study has portrayed the SA mediated defense mechanism involved in the induction of resistance in finger millet plants against blast pathogen *M. grisea* by using the *P. aeruginosa* isolates as inducers for seed priming.

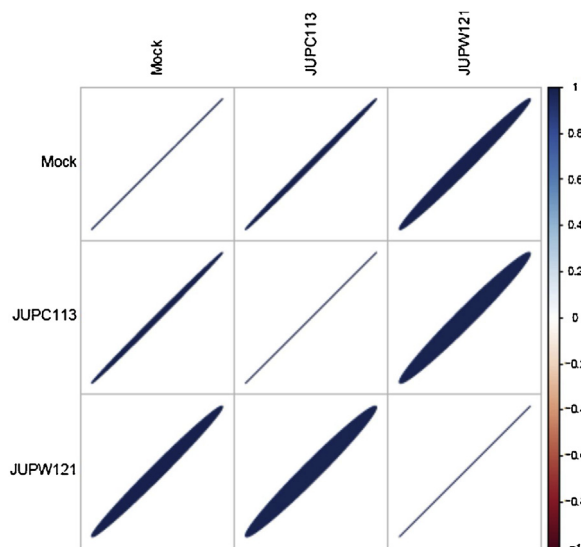


**Fig. 8.** Heatmap cluster among primed and non-primed plants after 0, 6 and 12 hours post-inoculation (hpi). The scale indicates: red color for higher and blue color for lower expression in comparison with the mock control (0 h) plants. Scale is the mean values of Log2 after normalization (n = 4).



**Fig. 9.** Plots of principal component (PC1 and PC2) results are obtained from relative gene expression and defense enzymes expressed in primed and mock plants ( $p < 0.05$ ).





**Fig. 10.** Pearson's correlation coefficient (PCC) analysis of relative gene expression and defense enzymes in primed and mock plants ( $p \leq 0.05$ ).

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## Author contributions

Experiment design and monitor: BSK, SJ, VKG. Performed the experiments: SVP. Data compilation: SVP, BSK, S-il, DPS, SJ. Supply of reagents/consumables: SS, BSK. Manuscript writing: SVP, BSK, HGP, SDB, SS, VKG, SJ. Preparation of Figures/Graphs: BSK, SJ. All the authors read and approved the manuscript.

## Ethical approval

This research does not involve human participants or animals.

## Declaration of Competing Interest

All the contributing authors here with confirm no conflicts of interest connected to this work.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2020.e00484>.

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