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Elucidating the immunity-pathogenesis nexus in rice and *Helminthosporium oryzae*: Insights from gene expression profiling and phytohormone analysis**

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Abstract. Rice (*Oryza sativa* L.) is a major global staple food and a key crop in Pakistan, however, it is increasingly being threatened by climate change and biotic stresses such as brown leaf spot (BLS). We investigated the role of plant growth regulators in enhancing rice resistance to *H. oryzae* in four newly developed rice lines and one local variety for resistance to *Helminthosporium oryzae* by analyzing plant growth regulators and gene expression in young plants after pathogen attack. Salicylic acid (SA) levels in infected plants increased significantly, with the highest levels recorded at 48 hpi in V_3, followed by V_1. Abscisic acid (ABA) and jasmonic acid (JA) levels also increased post-infection, with V_3 showing the highest increase for both hormones. Additionally, the expression of defense marker genes *OsWRKY*24, -30, -45, and -53 was upregulated following infection. *OsPR*1*, OsSIPK, OsPLDα*1, and PPO, demonstrated heightened expression, with notable peaks in V_1, V_3, and V_4. ET biosynthetic gene *ACS*2 alongside elevated SA corresponded with increased expression of *PR*1 and *LOX*3. Furthermore, *OsERF*3 levels significantly increased across all infected rice lines, particularly in V_3, suggesting a coordinated defensive response. These results revealed that SA, along with specific defense-related genes, crucially mediates rice response to *H. oryzae* infection, with line V_3 showing the most robust resistance among the tested rice lines. Our findings support the fact that pathogens manipulate hormone signaling pathways and relative expression of genes to disrupt and evade plant defenses.

Keywords: disease, *Helminthosporium oryzae*, immunity, molecular regulation, rice

1. INTRODUCTION

Rice (*Oryza sativa* L.), along with maize and wheat, accounts for most of the global food production (Akbar *et al.*, 2025a). Rice cultivation represents around 10% of Pakistan's total cultivated land, with Basmati rice varieties being particularly important commercially due to their superior grain quality (Bashir *et al.*, 2007). Pakistan's traditional rice farming system is distinguished by puddling, abundant water resources, and high energy demands (Matloob *et al.*, 2022). Biotic and abiotic stresses are two significant constraints that considerably reduce agricultural output (Cohen and Leach, 2019). Rice growth and productivity are increasingly being threatened by climate change as well as by pest and pathogen attacks (Fahad *et al.*, 2019). Rice disease management relies on resistant varieties, cultural practices, and chemical or biological controls (Akbar *et al.*, 2025b). Plants have evolved complex defense mechanisms to protect themselves from these stressors. The primary biotic stresses affecting rice production and quality include bacterial leaf blight, sheath blight, brown leaf spot (BLS), false smut, brown planthopper, and yellow stem borer (Singh *et al.*, 2020). Brown leaf spot, a fungal disease in rice crops, is caused by *Helminthosporium oryzae* (Sunder *et al.*, 2014). This disease has been documented in nearly all rice-growing countries, including Japan, India, China,

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Pakistan, and Thailand (Khalil *et al.*, 2014). Typically, BLS infection significantly reduces grain weight and quality and inhibits the growth of tillers (Kamal and Mia, 2009).

Research on rice defense and stress responses has highlighted the regulation of various transcription factors (TFs), genes, proteins, and secondary metabolites that respond to diverse challenges throughout the plant life cycle, including pathogen attacks (Akbar *et al.*, 2023; Younas *et al.*, 2023). Different TF families regulate plant growth, development, stress responses, and immunity, with members acting as positive or negative regulators against biotrophic, hemi biotrophic, and necrotrophic pathogens (Yuan *et al.*, 2019). Plants contain around 58 transcription factor families, *e.g.*, *AP2/ERF, bHLH, MYB, NAC, WRKY,* and *bZIP* playing critical roles in responses to both biotic and abiotic stresses (Noman *et al.*, 2019; Wang *et al.*, 2022). As master regulators of plant defense, these transcription factors are regulated at transcriptional, post-transcriptional, and post-translational levels (Noman *et al.*, 2017). Enhancing immunity against pathogens is particularly challenging in rice due to its complex self-defense mechanisms, and understanding the genetic foundations of these defense strategies is essential for developing rice varieties with improved resilience.

Plant responses to stressors involve intricate changes in the transcriptome, cellular structure, and physiological systems (Atkinson and Urwin, 2012). Plants typically use two primary immune strategies, *i.e.*, PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). These mechanisms function *via* signal transduction pathways that regulate key proteins, including transcription factors, protein kinases, and pathogenesis-related (PR) proteins (El Hadrami *et al.*, 2011; Guttman *et al.*, 2014). Transcription factors (TFs) are crucial regulators of plant processes, affecting metabolism, growth, and defense either positively or negatively (Choi *et al.*, 2020). For example, *OsWRKY*45 in rice acts as a master regulatory protein in coordination with *OsNPR*1 managing various SA responses (Sugano *et al.*, 2010). *OsNH*1 in rice is regarded as the closest homolog to *NPR*1 (Yuan *et al.*, 2007). Numerous defense-activated genes rely on *OsWRKY*45, including PR genes and defense-related TFs such as *OsWRKY*70 (De Vleesschauwer *et al.*, 2013). Similarly, *OsBIERF3* enhances immunity against *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *oryzae* in rice but reduces tolerance to cold stress (Hong *et al.*, 2022).

Both PTI and ETI activate signaling pathways involving MAP kinases (MAPKs), reactive oxygen species (ROS), SA, JA, ethylene (ET), and other phytohormones. These pathways lead to the production of antimicrobial compounds, secondary metabolites, and callose deposition and also trigger programmed cell death in response to stress. Phytohormones are essential not only for plant growth and development but also for activating plant immune responses (De Vleesschauwer *et al.*, 2014). Historically, research on the defense-related roles of growth regulators suggests that plant vigor and immunity are influenced by complex crosstalk involving changes in phytohormone levels, transcriptional control, and the lifestyles of invading pathogens (Aerts *et al.*, 2021; Salem *et al.*, 2021). Phytohormones regulate plant responses to external stimuli through intricate monitoring systems. Observations in model plant species show that changes in phytohormones (Jan *et al.*, 2020) along with the synthesis and function of SA, ABA, and JA during pathogen attacks are crucial in regulating disease-signaling pathways. Plants respond to microbial invasions by producing a complex blend of SA, JA, and ABA, depending on the infection strategy and lifestyle of the pathogens. SA is often effective against biotrophic diseases, which feed on living plant tissues, while JA-dependent defenses are more effective against necrotrophic infections, which cause cell death (Bari and Jones, 2009).

Plant defense systems are essential for resisting diseases and pests that threaten crop yield and quality (Upadhyay *et al.*, 2025). To address food safety and security challenges, research is aiming to uncover the mechanisms underlying plant resistance, with a focus on understanding how plants endure biotic and abiotic stresses. We, therefore, hypothesized that the defense response of rice to *H. oryzae* infection involves distinct changes in gene expression and phytohormone levels, which may vary among different rice lines. Plant growth regulators play a crucial role in modulating rice immunity and resistance to *H. oryzae*. Hence, this study aimed to investigate the relationship between rice immunity and the pathogenesis of *H. oryzae* by analyzing gene expression profiles and phytohormone changes across four newly developed rice lines and a standard variety.

2. METHODOLOGY

The experiment was conducted to evaluate rice lines for their susceptibility and resistance against *H. oryzae*. Four newly developing rice lines Sakh, Campena, Kharamana, and Binicol and one local variety (KSK-282) were grown to unveil their defense responses to *H. oryzae* infection. We assessed the particular role of plant growth regulators and defense marker genes at different time points. Leaves of 21-day-old *Oryza sativa* plants were collected at three time points, *i.e.*, 12, 24, and 48 h post infection (hpi). In the meantime, leaves of healthy plants at the same stage were also taken as control samples. After collection, all samples were immediately placed in liquid nitrogen and kept in a refrigerator at -80 °C, following the method of (Huang *et al.*, 2015; Su *et al.*, 2020).

2.1. Determination of salicylic acid

Standard stock solutions of SA and JA were prepared in methanol. Methanol was used to dilute the corresponding volume of the standard stock solutions to create mixed working solutions. The working and stock solutions were both kept in a freezer at -20°C in the dark. The leaf samples were kept at room temperature in liquid nitrogen. After the liquid nitrogen was evaporated, 1.0 g of the samples were preciμsely weighed and placed in a 50 mL centrifuge tube. Then, 10 mL of 80% cooled methanol (pH 2.5/3.0) and 10 μL internal standard of dihydro jasmonate (DHJA) were added. After homogenizing the mixture for 2 min at 6000 rpm, it was kept overnight at 4°C. The next day, 10 mL of ethyl acetate was added to the tube centrifuged for 10 min at 10000 rpm at 4°C. The tube was again centrifuged for 10 min at 10000 rpm (4°C) after adding 10 mL of ethyl acetate. After collecting the supernatant, 0.2 g of graphitized carbon blacks (GCB) and 0.6 g of primary secondary amine (PSA) were added and the mixture was vortexed for 1 min.

2.2. GC-MS condition

The mixture was transferred into an Agilent 6890N-5973i GC/MS system equipped with a DB-5MS (UI) chromatographic column (30 m × 0.25 mm × 0.25 μm). The injection temperature and volume were calibrated to 280°C and 2 μL, correspondingly; 1.1 mL min^{-1} was the helium flow rate. The starting column temperature was set at 70°C for 4 min. After that, it increased to 300°C at a rate of 10°C min^{-1} for 2 min. After that, it increased to 340°C at a rate of 5°C min^{-1} and was maintained until the analysis was completed. After electron ionization at 70 eV, quantification was carried out in the selected-ion monitoring (SIM) mode with a dwell period of 0.3 s. The source and quadrupole temperatures were adjusted at 230 and 150°C, respectively. Mass data were obtained within the range of m/z 40 to m/z 500.

2.3. Abscisic acid estimation

In order to estimate ABA, leaves were ground using a pestle and mortar in an extraction solution that contained 2% glacial acetic acid and 80% methanol following (Hsu and Kao, 2005). Plant pigments and other non-polar substances that may interfere with the immunoassay were eliminated from the extracts by first passing them through C18 cartridges and a polyvinylpyrrolidone column. Prior to the enzyme-linked immunosorbent assay (ELISA), the evaluates were resuspended in Tris-buffered saline after being vacuum evaporated until they were completely dry. ELISA was used to measure ABA (Walker-Simmons, 1987) using an ABA immunoassay detection kit (PGR-1), Sigma Chemical Co. (St. Louis, MO), which was specific for (þ)-ABA.

2.4. Extraction and quantitation of JA

In accordance with Gundlach *et al.* (1992), after being cultivated in the pathogenic conditions, plant tissue was frozen in liquid nitrogen. As internal standards, 100 ng of 9,10-dihydrojasmonic acid methyl ester and 100 ng of 9,10-dihydrojasmonic acid (obtained from JA methyl ester by catalytic hydrogenation with Pd/charcoal) were added as soon as the frozen cells were defrosted in 30 mL of ethanol. After 1 min of sonication, the samples were divided, and 30 mL ethanol was used to extract the tissue a second time. After drying the ethanolic extracts at 400°C, the residue was dissolved in 50 mL of water. The mixture was extracted using 50 mL of $CHCl_3$ and 3 mL of 12M HCO (hydrogen carbonate – a conjugate base of carbonic acid and a conjugate acid of carbonate) were added to acidify it. After dissolving the left over in 100 μL methanol, 1 mL of excess diazoethane in ether was added. A silica solid-phase extraction column (J. T. Baker Bakerbond SPE silica gel, 500 mg, 3 mL) was used to hold the residue after the sample was dried at room temperature for 30 min. 5 mL of n-hexane were used to wash the column, and 7 mL of n-hexane/diethyl ether, 2:1 (vol/vol), was used to evaluate the sample. The material was allowed to dry completely before being dissolved in 20 μL of methanol and subjected to gas chromatography/mass spectrometry (GC/MS) analysis. This involved using a Varan 3400 gas chromatograph connected to a Finnigan MAT quadrupole SSQ 700 mass spectrometer; a J and W Scientific DB-5 (30 x 0.25 mm) column with a linear flow rate of 23 cm s^{-1}; a temperature gradient of 50°C for 1 min, 50-160°C for 30 °C min^{-1}, 160-200°C at 5°C min^{-1}, 200-290°C at 30°C min^{-1}, 290°C for 5 min. The methyl ester was hydrolyzed to produce JA. The methyl esters of JA and 9,10-dihydro-JA were found and quantified. For JA, the fresh weight detection limit was almost 0.5 ng g^{-1}.

2.5. RNA Extraction and cDNA synthesis

2.5.1. Sample collection for RNA extraction

To extract RNA, sterilized tubes (2 mL) were labeled and used. Each tube contained three little steel balls. Subsequently, leaf samples from control and infected plants at different time points were placed inside these tubes and submerged in liquid nitrogen instantly. Following collection, all samples were kept in an ultralow refrigerator at -80°C until RNA was extracted.

2.5.2. Procedure of RNA Extraction

The TissueLyser equipment was used to run stored samples for 3 min at 30 cycles per minute. The crushed material was transferred to fresh tubes. After adding 1 mL of trizol, the tubes were vortexed for 2 min. The samples were centrifuged for 15 min at 12000 × g at 4°C. 200 μL of chloroform was added after 900 μL of the supernatant was transferred into fresh tubes (1.5 mL) and allowed to remain at room temperature for 5 min. The tubes were shaken vigorously for 15 s and then placed at room temperature for an additional 5 min. The samples were centrifuged at 12000 × g and 4°C for 15 min. 500 μL of the supernatant without debris was transferred into new tubes. The tubes were again placed for 2 min at room temperature. An equal volume of 500 µL of isopropyl alcohol was added to the samples and gently stirred. The samples were centrifuged for 15 min at 4°C and 12000 × g. After adding 1 mL of ethanol (75%) to each sample to wash the RNA pellets, the samples were centrifuged once more for 5 min at 4°C and 7500 ×g. The liquid was carefully discarded, but the pellets at the bottom were preserved. After 15 min of laminar flow drying, the RNA pellets were checked to make sure the ethanol had evaporated. After that, RNA was dissolved in 20 µL of RNAase-free water and kept at -80°C. cDNA was synthesized and used for qRT-PCR analysis.

2.5.3. Quantitative real-time-PCR (qRT-PCR)

qRT-PCR was used to determine the relative transcription levels of the chosen genes using particular primers (Supplementary Table S3) in accordance with the Bio-Rad RT-PCR system (Foster City, CA, USA) and the SYBR Premix Ex Taq II system (TaKaRa) manufacturer's handbook. The RT-PCR and RNA preparation were done in accordance with earlier research. 4 replicates for each treatment were arranged for recording data following the Livak method (Livak and Schmittgen, 2001). Recorded data were examined and represented for each gene as a normalized relative expression level ($2^{-\Delta\Delta C}T$) (Noman *et al.*, 2018; 2019).

2.6. Statistical analysis

The data were analyzed statistically using one-way analysis of variance (ANOVA), with results presented as mean ± standard error (SE). Comparisons between two groups were conducted and Tukey's post-hoc test was applied for multiple comparisons. A P-value below 0.05 was regarded as statistically significant.

3. RESULTS

3.1. Plant growth regulators

Plant growth regulators play a vital role in rice resistance against pathogens and pests. Therefore, we focused on plant growth regulators at different time points to unravel rice resistance to *H. oryzae* in four newly developing rice lines and a local variety. Under pathogen infection, the level of ABA was much higher in V_3 than in the others. At 12 hpi, the levels of ABA exhibited a remarkable increment in V_3, compared to the control. All rice types showed a non-significant behavior for ABA under non-infection conditions (Fig. 1).

At 24 hpi, a statistically significant increment in ABA levels was observed in V_2, V_3, and V_4. Like at 12 hpi, the levels of ABA in all rice types were non-significantly related under the control conditions at 24 hpi. Meanwhile, the significant difference for ABA lasted from 12, 24, to 48 hpi. Although at 48 hpi the ABA levels increased in response to *H. oryzae* attack, there was no substantial increase in ABA in V_5. In V_1, V_3, and V_4, the ABA levels increased. Additionally, only V_5 showed a comparative increase in ABA under the control conditions (Fig. 1).

Under pathogen infection, the accumulation of SA in rice showed significant variation among all studied types at different time points. Although optimal variation in SA levels was recorded among the control rice plants, the significance of the variation in SA levels was comparatively higher in all plants experiencing *H. oryzae* attack. At 12 hpi, the SA levels in V_1, V_3, and V_4 displayed a clear increment, compared to the control. After 24 hpi, a statistically significant increase in the SA levels was observed in the rice lines but there was no significant difference from the SA levels recorded after 12 hpi, with the maximum increase recorded in V_3 followed by V_1 and V_5. In contrast to the 12 hpi time point, the SA levels in all rice types were non-significantly related under the control conditions at 24 hpi. Moreover, the SA levels increased at 48 hpi in response to the pathogen attack in all rice lines and the analyzed variety. A substantial increment in SA in all rice types was noticed in V_1, V_2, V_3, V_4, and V_5. A minimum increase in SA was recorded in V_3, compared to the control. Additionally, all rice types showed a non-significant or least significant relationship with each other for SA under control conditions (Fig. 1).

Upon pathogen infection, V_3 exhibited a significantly higher level of JA, compared to the other varieties at different time points. At 12 hpi, although the highest JA was found in V_3, the JA levels in V_5 showed a noteworthy surge, compared to the control. All rice types displayed a minimal significant JA concentration in the control conditions. By 24 hpi, a statistically significant increase in the JA levels was observed in the rice lines, with the maximum increase recorded in V_3 followed by V_1 and V_4. In contrast to 12 hpi, the JA levels in all rice types were non-significantly related under the control conditions at 24 hpi. Furthermore, the significant difference in the JA levels persisted from 12 to 24 hpi and extended to 48 hpi. Although the JA levels increased after 48 hpi in response to the *H. oryzae* attack, the increase in JA in V_1, V_2 and V_4 was not very substantial and showed similarity with values recorded at 12 hpi. For V_5, the JA levels increased at 12, 24, and 48 hpi. Additionally, all rice types showed a non-significant ($p \leq 0.05$) relationship for JA under the control conditions.

3.2. Relative expression of defense marker genes

To assess changes in the expression level of *OsACS*1, the 4 different rice lines and the local variety were exposed to *H. oryzae* attack at different time points under natural field conditions. No significant difference in the relative expression level of *OsACS*1 was observed in the rice plants at 12 hpi. In the control plants, changes in the relative expression of *OsACS*1 were noted. V_5 exhibited the lowest relative expression level of *OsACS1* among the non-infected plants. In turn, at 24 hpi, all rice plants displayed a highly significant relative expression level of *OsACS*1,

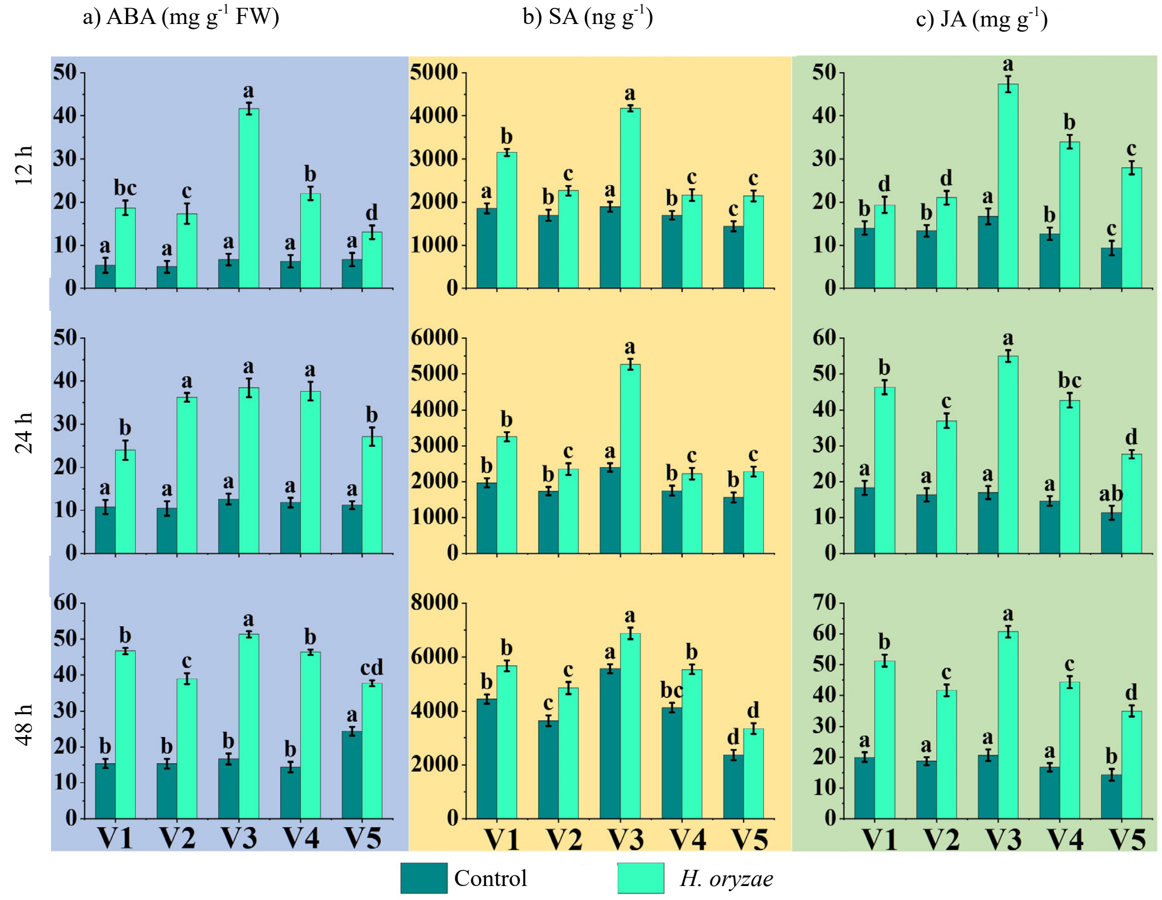

Fig. 1. Changes in plant growth regulators level at different time points are evaluated as: a) Abscisic acid (ABA, mg g^{-1} FW), b) Salicylic acid (SA, ng g^{-1}), and c) Jasmonic acid (JA, mg g^{-1}) in rice plants infected with *H. oryzae* compared to control plants. Significant variations across rice lines are represented by different letters above the bars. The lines with identical alphabets above the bars do not exhibit statistical significance (n = 5, $p \leq 0.05$). V_1 = Sakh, V_2 = Campena, V_3 = Kharamana, V_4 = KSK-282, V_5 = Binicol).

which reached its maximum value in V_3. A non-significant difference in *OsACS*1 activity was noted in V_1 and V_5 at this time point, and both of these lines were behind the others in the relative expression of *OsACS*1. Overall, a highly significant difference was seen between the control and the *H. oryzae*-infected plants (Fig. 2).

At 12 hpi, a significantly increased expression level of *OsACS*2 was recorded in all rice types after the exposure to *H. oryzae*, compared to the control. Our findings showed a significant difference in all rice lines for this attribute. V_3 exhibited the highest expression level of *OsACS*2, followed by V_2 and V_4, while V_1 showed a minimum relative expression level against the pathogen at 12 hpi. In contrast, in the non-inoculated conditions, there was no significant difference in their values. At 24 hpi, an almost similar trend was shown, but all values were increased, compared to 12 hpi. V_3 showed the maximum value, followed by V_2 and V_4. Contrarily, in the non-inoculated plants, a non-significant gene expression level was observed. V_1 and V_5 did not differ statistically significantly (Fig. 2).

At 12 hpi, no significant difference in the relative expression level of *OsAOS*1 was observed in the rice plants. In the control plants, changes in the relative expression of *OsAOS*1 were noted. V_5 exhibited the lowest relative expression level of *OsAOS*1 among the non-infected plants. In turn, at 24 hpi, all rice plants displayed a highly significant relative expression level of *OsAOS*1, which reached its maximum value in V_3. A non-significant behavior in *OsAOS*1 activity was noted in V_1 and V_5 at this time point, and both lines were behind the others in the relative expression of *OsAOS*1. Overall, a highly significant difference was seen between the control and the *H. oryzae*-infected plants (Fig. 2).

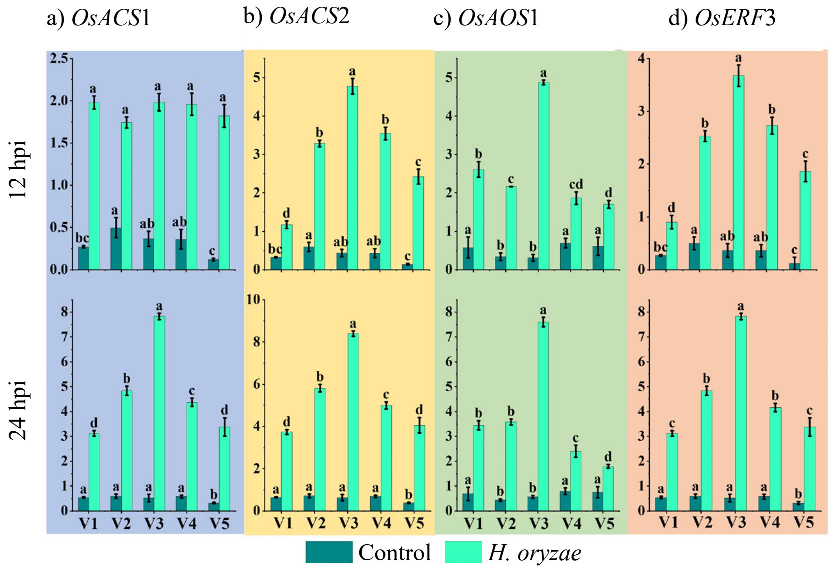

Fig. 2. Relative expression level of: a) *OsACS*1, b) *OsACS*2, c) *OsAOS*1, d) *OsERF*3 at different time points of 12 hpi, and 24 hpi in *H. oryzae* inoculated and non-inoculated rice plants. Alphabets above the bars depict significant difference between rice lines. Bars sharing identical alphabets denote statistically non-significant behavior (n = 5, $p \leq 0.05$). V_1 = Sakh, V_2 = Campena, V_3 = Kharamana, V_4 = KSK-282, V_5 = Binicol.

For investigating the role of *OsERF*3 in rice defense against *H. oryzae* infection, we recorded changes in the relative expression level of *OsERF*3 in rice plants under control and pathogen attack. At 12 hpi, significant differences in the relative expression level of *OsERF*3 were seen in the rice plants. The highest expression level was found in V_3, followed by V_2 and V_4. In the control plants, changes in the relative expression of *OsERF*3 were noted, but these were not highly significant. In turn, at 24 hpi, all rice plants displayed a non-significant relative expression level of *OsERF*3 under the control conditions. Among the *H. oryzae*-infected plants, highly significant changes were observed in almost all rice plants. V_3 showed the highest relative expression level of *OsERF*3, followed by V_2 and V_5 (Fig. 2).

Compared to the non-infected plants, our study demonstrated that the significantly enhanced expression of *OsLOX*2 at 12 hpi in V_2 was followed by V_3 and V_4, and these all contributed to rice plant defense response. Despite the enhanced expression, this increase was non-significant between the V_1 and V_5 rice plants experiencing the pathogen attack. All control plants presented a non-significant relationship for the relative expression of *OsLOX*2 at 12 hpi. At 24 hpi, a slightly variable trend was shown, but the relative expression in all plants was increased, compared to 12 hpi. V_3 displayed the highest relative expression level *versus* the control plants as well as the infected plants and was followed by V_1 and V_4 (Fig. 3).

Our findings showed that, under the pathogen infection, V_3 exhibited a significantly higher expression level of *OsPLDα*1, compared to the others at both time points. At 12 hpi, the *OsPLDα*1 expression levels in V_1 and V_5 showed a noteworthy surge, compared to the control. All rice types in the control conditions displayed a minimal significant relative expression level of *OsPLDα*1. By 24 hpi, a statistically significant increase in the *OsPLDα*1 expression levels were observed in the rice lines, with the maximum increase recorded in V_3, followed by V_1 and V_2. In contrast to 12 hpi, the *OsPLDα*1 expression levels in all rice types under the control conditions were non-significant. But it was overall non-significant. Furthermore, the *OsPLDα*1 expression level increased after 24 hpi in response to the *H. oryzae* attack. The highest increment was observed in V_3, followed by V_1 and V_2, as they were increased, compared to the non-inoculated plants. Additionally, all rice types showed a non-significant ($p \leq 0.05$) relationship for the *OsPLDα*1 expression level under the control conditions (Fig. 3).

Our research revealed that the *H. oryzae* attack influenced the expression of *OsLRR*1. Although the pathogen attack caused an increase in the relative expression level of *OsLRR*1, yet the highest value of expression was noticed in V_3, followed by V_2 at 12 hpi. The *OsLRR*1 expression in the infected plants was significantly different from that in the control plants. In contrast, a nearly same trend was seen at 24 hpi, but all values were increased from 12 hpi. Compared to the control/non-infected plants, V_3 had the greatest relative expression level followed by V_2, but V_1 and V_5 as well as V_2 and V_4 were not highly significantly different. In turn, in the control plants, V_2 and V_4 showed the highest values, followed by V_1. The expression of *OsLRR*1 was not significantly different at either time point (Fig. 3).

Under the pathogen infection, the relative expression level of *OsMPK*4 was much higher in V_3 than in the others. At 12 hpi *H. oryzae* infection, the levels of *OsMPK*4 exhibited a remarkable increment in V_3, compared to the control. All rice types showed a non-significant behavior

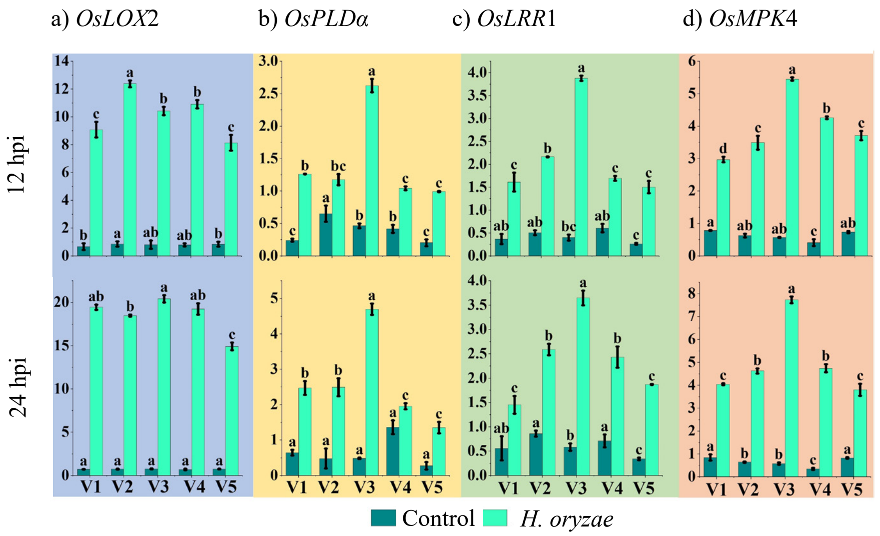

Fig. 3. Relative expression level of: a) *OsLOX*2, b) *OsPLDα*, c) *OsLRR*1, d) *OsMPK*4 at different time points of 12 hpi, and 24 hpi in *H. oryzae* inoculated and non-inoculated rice plants. Alphabets above the bars depict significant difference between rice lines. Bars sharing identical alphabets denote statistically non-significant behavior ($n = 5$, $p \leq 0.05$). V_1 = Sakh, V_2 = Campena, V_3 = Kharamana, V_4 = KSK-282, V_5 = Binicol.

for *OsMPK*4 under the non-infection conditions. Contrary to 12 hpi, a decrement in the *OsMPK*4 levels was observed at 24 hpi in V_2, V_3, and V_4. Like 12 hpi, the expression levels of *OsMPK*4 in all rice types were non-significantly related under the control conditions (Fig. 3). To detect dynamic changes in the relative expression level of *OsNPR*1, the rice plants were infected with *H. oryzae* and data were recorded at different time points. At 12 hpi, the highest expression level of *OsNPR*1 was observed in V_4 and V_1, compared to the other plants. In the control plants, no significant changes were noticed in their values (Fig. 4), whereas at 24 hpi, a similar pattern was observed in the relative expression level of *OsNPR*1, which reached its maximum in V_1 and V_4. The lowest *OsNPR*1 activity was noted in V_3, but it was also higher than its counterpart grown in the control. Overall, a significant increment was noticed after 24 hpi compared to 12 hpi (Fig. 4).

Consistent with the higher abundance of PPO transcripts in the pathogenic conditions, all rice lines exhibited increases in the PPO expression level, compared to that of the non-inoculated control plants. At 12 hpi, the highest relative expression level was recorded in V_1, V_3, and V_4. Differential and significantly higher relative expression levels of PPO were evident in the infected plants, compared to the control plants, while consistently low levels of endogenous PPO expression were present in rice types

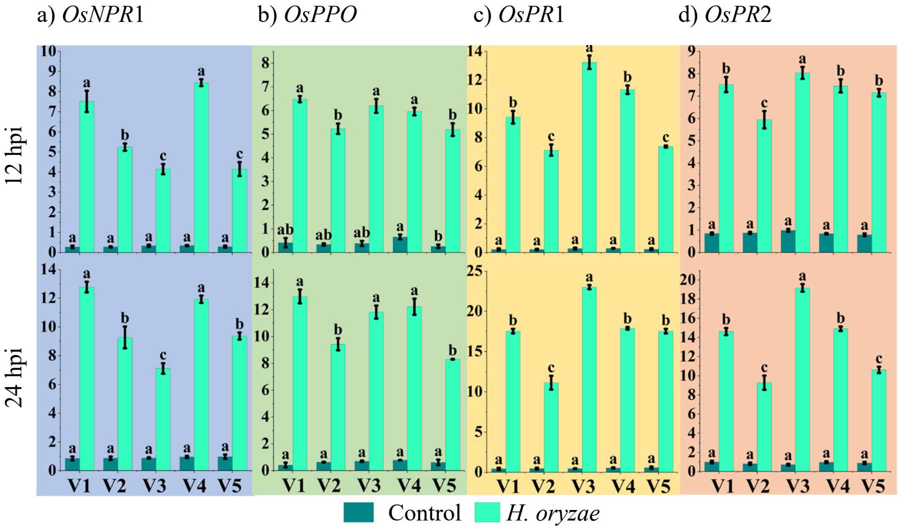

Fig. 4. Relative expression level: a) *OsNPR*1, b) *OsPPO*, c) *OsPR*1, d) *OsPR*2 at different time points of 12 hpi, and 24 hpi in *H. oryzae* inoculated and non-inoculated rice plants. Alphabets above the bars depict significant difference between rice lines. Bars sharing identical alphabets denote statistically non-significant behavior ($n = 5$, $p \leq 0.05$). V_1 = Sakh, V_2 = Campena, V_3 = Kharamana, V_4 = KSK-282, V_5 = Binicol.

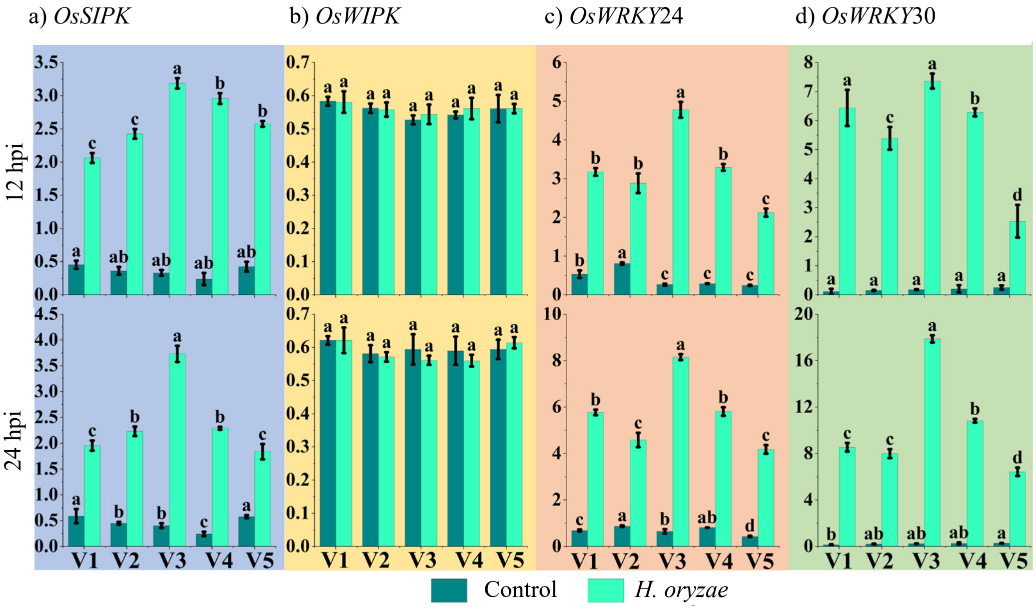

Fig. 5. Relative expression level of: a) *OsSIPK*, b) *OsWIPK*, c) *OsWRKY*24, d) *OsWRKY*30 at different time points of 12 hpi, and 24 hpi in *H. oryzae* inoculated and non-inoculated rice plants. Alphabets above the bars depict significant difference between rice lines. Bars sharing identical alphabets denote statistically non-significant behavior ($n = 5$, $p \leq 0.05$). V_1 = Sakh, V_2 = Campena, V_3 = Kharamana, V_4 = KSK-282, V_5 = Binicol.

grown in the non-inoculated conditions. After 24 hpi, the relative expression level of PPO reached its maximum in V_1, V_3, and V_4, likewise at 12 hpi, and declined thereafter in the non-inoculated plants (Fig. 4). During the pathogen infection, the expression of *OsPR*1 was significantly higher in V_3, compared to the others. After 12 hpi, V_1, V_3, and V_4 exhibited a substantial increase in the *OsPR*1 expression levels, compared to the control. All rice lines showed no significant changes in *OsPR*1 expression under the non-infection conditions. In contrast to 12 hpi, a noticeable increase in the *OsPR*1 levels was observed. V_2 exhibited the lowest *OsPR*1 levels. Similarly, the expression levels of *OsPR*1 in all rice types were non-significantly related under the control conditions (Fig. 4).

At 12 hpi, a significantly increased expression level of *OsPR*2 was recorded in all rice types after the exposure to *H. oryzae*, compared to the control. V_3 exhibited the highest expression level of *OsPR*2, followed by V_1, V_3, and V_5, while V_2 showed a minimum relative expression level against the pathogen at 12 hpi. In turn, in the non-inoculated conditions, there was no significant difference in their values, whereas an almost similar trend was shown at 24 hpi, but all the values were increased, compared to 12 hpi. V_3 showed the maximum value, followed by V_1 and V_4. Contrarily, in the non-inoculated plants, a non-significant gene expression level was observed (Fig. 4).

At 12 hpi, the *OsSIPK* expression level in V_3, V_4, and V_5 displayed a clear increment, compared to the control. After 24 hpi, a statistically significant increase in the *OsSIPK* relative expression levels was observed in the rice lines, with the maximum increase recorded in V_3, followed by V_2 and V_4. In contrast to 24 hpi, the *OsSIPK* levels in all rice lines were also significantly changed, as V_1 and V_5 exhibited the highest relative expression level while V_4 recorded as the lowest expression level (Fig. 5).

The expression of *OsWIPK* at 12 hpi in V_3 followed by V_4 showed a remarkable enhancement of the rice plant defense response, compared to the control plants, demonstrating a significant increase in resistance against pathogen attack. At 24 hpi, an almost similar trend was shown, but all values were increased, compared to 12 hpi. V_3 displayed the highest increase in the relative expression level. Additionally, all rice lines showed a minimal significant relationship with each other for the *OsWIPK* relative expression level at both time points (Fig. 5). Our study revealed that the expression of *OsWRKY*24 at 12 hpi in V_3 followed by V_1, V_4, and V_2 showed a remarkable enhancement of the rice plant defense response, compared to the control plants, demonstrating a substantial increase in resistance against pathogen attack. At 24 hpi, an almost similar trend was shown, but all values were increased, compared to 12 hpi. V_3 displayed the highest increase in the relative expression level, compared to the control/ non-infected plants, followed by V_1 and V_4 (Fig. 5).

To detect dynamic changes in the expression level of *OsWRKY*30, TF in 4 different rice lines and the local variety were assessed at different time points under natural field conditions and under *H. oryzae* attack. At 12 hpi, the highest expression level of *OsWRKY*30 was observed in V_3, followed by V_1 and V_4, compared to the control plants (Fig. 5). The relative expression level of *OsWRKY*30

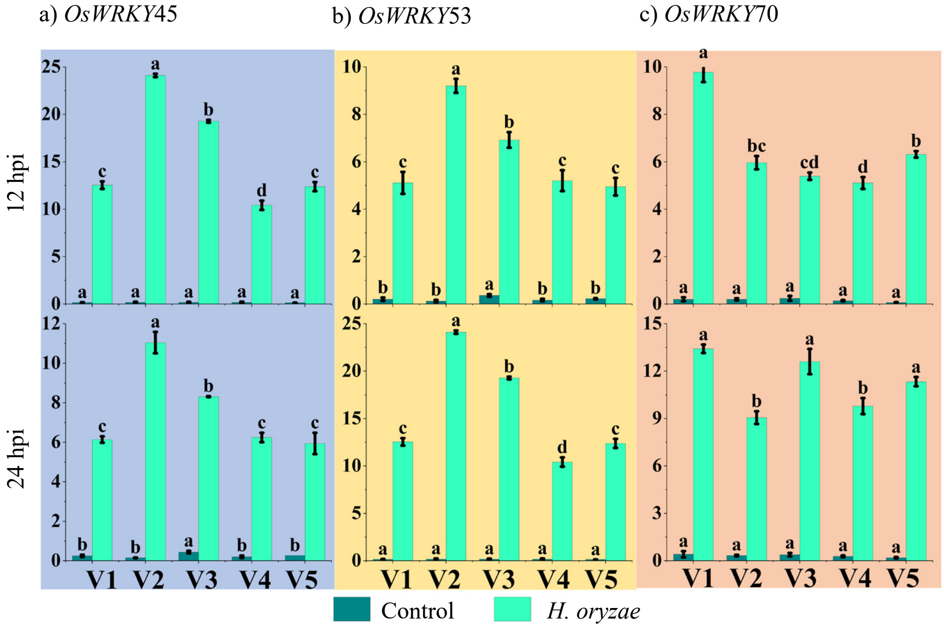

Fig. 6. Relative expression level of TFs at different time points of 12 hpi, and 24 hpi: a) *OsWRKY*45, b) *OsWRKY*53, c) *OsWRKY*70 in *H. oryzae* inoculated and non-inoculated rice plants. Alphabets above the bars depict significant difference between rice lines. Bars sharing identical alphabets denote statistically non-significant behavior ($n = 5$, $p \leq 0.05$). V_1 = Sakh, V_2 = Campena, V_3 = Kharamana, V_4 = KSK-282, V_5 = Binicol.

reached its maximum in V_3 at 24 hpi. The lowest *OsWRKY*30 activity was noted in V_5, but it was also higher than its counterpart grown in the control (Fig. 5).

Our investigation revealed a dynamic modulation in the *OsWRKY*45 expression in response to the pathogen. At 12 hpi, a notable increase in the relative expression level was observed after the pathogen attack in all rice lines. V_2 exhibited the highest value, followed by V_3. In contrast, the lowest relative expression level was noted in V_4. All the control plants showed non-significance. The experimental evidence presented a remarkable increase in their values at 24 hpi. V_2 displayed the highest value, followed by V_3 among all lines, although the values in the other lines were not significant. In the non-infected plants, V_3 showed the maximum value among all control plants, while the values in the other lines are almost non-significant (Fig. 6). At 12 hpi, the relative expression level of *OsWRKY*53 was much higher in V_2, followed by V_3, compared to the control plants. All rice types showed a non-significant behavior for *OsWRKY*53 under the non-infection conditions. At 24 hpi, a statistically significant increment in the *OsWRKY*53 levels was observed in V_2 and V_3. Like 12 hpi, the levels of *OsWRKY*53 in all rice types were non-significantly related under the control conditions (Fig. 6).

Under the pathogen infection, the relative expression level of *OsWRKY*70 in rice showed significant variation among all studied lines at different time points. Although optimal variation of the expression level of *OsWRKY*70 was recorded among rice plants in the control, the significance of the variation in the relative expression was comparatively higher in all plants experiencing the *H. oryzae* attack. At 12 hpi, the relative expression level of the *OsWRKY*70 gene in V_1, V_2, and V_4 displayed a clear increment, compared to the control. After 24 hpi, a statistically significant increase in the *OsWRKY*70 relative expression level was observed in the rice lines, but it was not significantly different from the *OsWRKY*70 relative expression level recorded after 12 hpi, with the maximum increase recorded in V_1, followed by V_3 and V_5. In contrast to 12 hpi, the relative expression levels in all rice lines were non-significantly related under the control conditions at 24 hpi. Moreover, this level increased at 48 hpi in response to the pathogen attack in all rice lines and the variety. The substantial increment in SA in all rice lines was seen in V_1, V_2, V_3, V_4, and V_5. Additionally, all rice lines showed a non-significant or the least significant relationship with each other under the control conditions (Fig. 6).

4. DISCUSSION

ET, SA, and JA are commonly referred to as phytohormones involved in biotic stress response. Hormonal signaling mediated by SA, JA, and ET is typically associated with plant defense mechanisms (Kazan and Lyons, 2014). JA and ET act antagonistically to SA. This leads plants to prioritize one defense pathway, either the JA/ET or the SA pathway, in response to pathogen invasion (Gorshkov and Tsers, 2022). For example, *Xanthomonas campestris* has been shown to increase SA levels in susceptible hosts

(O'Donnell *et al.*, 2001). The same phenomenon was also observed in this study, while an inverse pattern was noted for JA. Although variation in SA levels was recorded in the control rice plants, the significance of the SA variation was notably higher in all plants exposed to *H. oryzae,* indicating that SA rather than JA plays a more crucial role in defense. The accumulation of JA may be attributed to routine plant processes. This reduced defensive role of JA aligns with the findings of Zheng *et al.* (2012), who concluded that post-infection SA accumulation confers resistance to pathogenic bacteria in JA-insensitive plants. The antagonistic relationship between JA and SA is well-documented, with SA often inhibiting JA activity (Bostock, 2005). We observed that the activation of the SA-dependent defense pathway reduced disease symptoms. Additionally, SA signaling is proposed as a potential target for manipulation in *O. sativa*. Our findings provide evidence of the role of SA in plant-pathogen interactions, supporting the fact that pathogens manipulate hormone signaling pathways to disrupt and evade plant defenses. This interaction fine-tunes plant defense responses depending on the pathogen, as discussed by Eccleston *et al.* (2022), Grant and Jones (2009), Spoel and Dong (2024).

We also observed the highest ABA levels in V_3, suggesting a strategic response to manage stress and prime cells for potential challenges, consistent with findings from Mauch-Mani and Mauch, who noted that ABA plays a role in resistance by activating defense genes (Mauch-Mani and Mauch, 2005). Conversely, the decreased ABA levels in V5 indicated a weaker defense response, allowing greater pathogen impact on rice growth. Observed ROS and MDA values (Akbar *et al.,* 2025b) directly correlated with ABA quantification in this study, highlighting a link between stress tolerance and ABA functionality along with other physiological roles. Plant hormones interact within complex networks to balance responses to developmental and environmental signals, thereby minimizing fitness costs associated with defense. The molecular mechanisms that regulate these hormonal networks remain largely unclear. Additionally, pathogens manipulate hormone signaling pathways to disrupt and evade plant defenses.

The intricate regulatory networks in plants can initiate responses following pathogen invasion, with various genes becoming active during these processes. Identifying these genes could provide new insights into rice response to pathogens and reveal the complex interactions between signaling networks that determine resistance or susceptibility to diseases (Kong *et al.*, 2018). This study suggests that WRKY transcription factors (TFs), particularly *OsWRKY24*, -30, and -45, play a role in plant defense against pathogens, as indicated by their expression patterns in response to *H. oryzae* and fluctuations in endogenous SA levels. Altered rice responses in terms of *WRKY* TF expression following pathogen infection were associated with changes in phytohormone levels and the expression of *PR*1*, PR*2, and *ERF*3 genes. Together, these findings propose that *OsWRKY*24, -30, and -45 contribute to a positive feedback loop in an SA-ET dependent defense signaling pathway, thus mediating resistance to brown leaf spot disease. These transcriptional feedback loops allow plants to manage multiple processes, simultaneously leading to metabolic adjustments that culminate in a defensive response (Shen *et al.*, 2016).

Supporting our findings, a study by Xiao *et al.* (2021) revealed that post-pathogen expression of *GhTINY*2 promotes SA accumulation and signaling by directly activating *WRKY*51 expression, highlighting the *TINY*2-*SA*-*WRKY* crosstalk as a molecular switch coordinating growth and defense during stress conditions. Our results align with those of Eulgem and Somssich (2007) and Wei *et al.* (2013) who reported that various *WRKY* genes are rapidly induced by different pathogens. This induction triggers the expression of immunity-related genes, leading to adaptive responses and enhanced stress tolerance in plants (Dang *et al.*, 2014; Yoo *et al.*, 2014; Zhang *et al.*, 2019). The observed changes in the *OsPR*1 and *OsPR*2 expression in this study support these findings, suggesting that pathogen-induced expression of PR genes is directly correlated with resistance and differential responses of rice lines to BLS.

A key feature of plant immune responses to pathogenic microbes is the role of essential regulatory genes as mediators of various plant hormone signaling pathways. For instance, SA is crucial for systemic acquired resistance (SAR), a traditional defense response in plants involving PR gene activation, leading to broader microbial resistance (Durrant and Dong, 2004). Additionally, polyphenol oxidase (PPO) activity is often associated with SA production and hydrogen peroxide (H_2O_2) accumulation. In this study, elevated SA levels were correlated with *OsPPO* expression, and similarly, H_2O_2 production was closely linked with PPO transcripts. These observations are consistent with findings by Nawrocka *et al.* (2018) and Saleem *et al.* (2020), who found that *Rhizoctonia solani* infection pretreated with *Trichoderma atroviride* increased H_2O_2 and PPO levels in stressed cucumbers.

It has been reported that inhibiting pathogen- and wound-responsive MAPKs, specifically *SIPK* and *WIPK*, in tobacco results in an abnormal accumulation of SA upon injury and a decrease in JA levels (Seo *et al.*, 2007). This observation aligns well with our findings, which show a lack of significant expression of WIPK following *H. oryzae* infection. Similarly, the small variations in JA compared to SA observed in our study are also supported by previous reports. Additionally, our results are consistent with findings that disease-responsive genes are upregulated in *WIPK*-suppressed plants (Katou *et al.*, 2013). Furthermore, *ACS*2 and *ACS*6 have been identified as substrates of *MPK*3 and *MPK*6, contributing to pathogen-responsive ethylene production through the phosphorylation and stabilization of *ACS*2 and *ACS*6 proteins (Han *et al.*, 2010; Xu and Zhang, 2014). It has also been shown that several *ERF* transcription factors act as substrates for *MPK*3/*MPK*6. From the initial perception of a pathogen to the final defense response, plant metabolism undergoes shifts that influence the plant's ability to combat stress. Any disruptions in these metabolic shifts can compromise the entire defense mechanism. Therefore, the recorded changes in the transcript levels at both time points correlate positively with defense responses in the rice lines (Zhou *et al.*, 2009).

5. CONCLUSIONS

In conclusion, the coordinated increase in SA, ABA, and JA levels coupled with the upregulation of key defense-related genes underscores a complex and dynamic hormonal network that underlies pathogen resistance. Among the tested rice lines, V_3 exhibited the most pronounced hormonal induction and gene expression, highlighting its superior resistance capacity. The enhanced expression of *defense genes* suggests a synergistic activation of SA-dependent defense pathways. These findings reinforce the point that hormonal crosstalk is central in activating effective plant immune responses. Overall, these interactions can serve as potential targets for breeding rice varieties with improved disease resistance.

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Authors contributions. MUA: experimentation, analysis, first draft preparation; MA: design, graphics; MQ: analysis, first draft preparation; MKI: analysis, revision; MOA: statistical analysis; NMA: analysis, revision; AN: conceived idea: supervision. All authors have read and agreed to the published version of the manuscript.

Conflict of interest. Authors declare no conflict of interest.

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