Disclosure of Defensive Enzyme of BRRI dhan29 and IR 64 to *Pyricularia oryzae* Stress

A THESIS

BY

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Abstract

Rice blast disease, caused by *Pyricularia oryzae*, is a significant threat to global rice production, resulting in substantial yield losses. However, productivity losses due to rice blast disease caused by *Pyricularia oryzae* are significant, and detailed knowledge of the enzymatic responses linked to physiological resistance remains limited. This study examines the defensive bichemicals and enzymatic responses in two rice cultivars, BRRI dhan29 and IR 64, to *Pyricularia oryzae* stress. The study was done by using a factorial completely randomized design with three replications. Cultivars were inoculated with a virulent *P. oryzae* strain (RL-17) at the Plant Protection Laboratory, Khulna University. The results showed a consistent increase in SOD, PAL, and β-1,3-glucanase activities in both cultivars, with IR64 exhibiting significantly higher SOD activity from 72 to 120 hours post-inoculation, indicating enhanced enzymatic defense and greater stress resilience. LOX and chitinase activities were comparable between the two cultivars, suggesting similar stress responses mediated by these enzymes. Furthermore, IR 64 showed enhanced phenol and flavonoid accumulation, correlating with its elevated antioxidant capacity and reduced oxidative damage. ROS dynamics (superoxide and hydrogen peroxide) varied between the cultivars, with BRRI dhan29 exhibiting excessive ROS accumulation, potentially leading to oxidative stress, whereas IR 64 maintained a balanced ROS-scavenging system. Protein and sugar levels also increased significantly in IR 64, indicating better resource allocation for defense. IR 64 demonstrates superior resilience to *Pyricularia oryzae* stress, making it a promising resistant benchmark for breeding programs, while targeted improvements are needed to enhance the stress tolerance of the mega variety BRRI dhan29 for food security.

Keywords: Defense, Enzyme, BRRI dhan29, IR 64, *Pyricularia oryzae*, Stress, Biochemical compounds

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Chapter I

Introduction

Rice (*Oryza sativa)* is a cornerstone of global food security, serving as a staple food for more than half of the world's population. Over half of all people on the planet eat rice as their primary food. Annual production of milled rice is over 480 million metric tons. For the millions of people living in poverty in Asia, rice accounts for up to 50% of their daily calorie intake, making it essential for food security (Muthayya et al., 2014). Since the Green Revolution began, rice production has increased at record rates. However, rice continues to be one of the food products with the highest levels of trade protection. Rice is produced and consumed in large quantities by Asian communities.

The main crop in the agrarian economy of Bangladesh is rice. Over the years, the country has made significant advancements in rice farming techniques, yield optimization, and cultivation strategies. Despite challenges posed by overpopulation, Bangladesh has successfully attained self-sufficiency in rice production, ensuring food security for its people. This progress highlights the nation's commitment to enhancing agricultural practices and sustaining its rice-based economy (Mamun et al., 2021).

Three rice cultivars were issued by Bangladesh Agricultural University, 25 types by Bangladesh Atomic Agriculture Research Institute, and 115 variants by Bangladesh Rice Research Institute (BRRI). Among them BRRI dhan29 is a most popular mega variety is highly susceptible to blast disease(Hossain et al., 2017). On the other hand, IR 64 is observed as blast resistance (Ahmed et al., 2022).

Cereal crops are the foundation of global food security. They face a constant threat from various devastating diseases. Among these, blast disease, caused by the fungal pathogen *Magnaporthe oryzae* (anamorph*: Pyricularia oryzae),* stands out as a major menace particularly in rice-growing regions. It can cause significant yield losses, impacting the livelihoods of millions of people who depend on rice for sustenance. The disease affects all aerial parts of the rice plant, including leaves, nodes, and panicles, disrupting photosynthesis and nutrient translocation (Shahriar et al., 2020). The severity of blast disease can vary depending on the fungal strain, host genotype, and environmental conditions. Favourable environmental conditions, such as high humidity and moderate

temperatures, exacerbate the severity of rice blast, leading to widespread outbreaks and productivity losses ranging from 10% to 90% (Hossain et al., 2017; Khan et al., 2016).

Favourable conditions for the blast disease include high humidity (above 90%), moderate temperatures ranging from 20°C to 28°C, and extended periods of leaf wetness, often resulting from dew or frequent rainfall (Shahriar et al., 2020). These climatic conditions are prevalent during the monsoon and post-monsoon seasons in Bangladesh, particularly in regions where irrigation practices create additional moisture. Dense planting, poor airflow, and excessive use of nitrogen fertilizer further exacerbate disease development by creating a microenvironment conducive to fungal growth and spore dispersal. The tropical and subtropical climate of Bangladesh provides ideal conditions for the rapid spread of rice blast, especially during critical growth stages like the tillering and panicle initiation phases, leading to substantial yield losses if not managed effectively (Kirtphaiboon et al., 2021).

Effective management of rice blast disease requires an integrated approach combining cultural, chemical, and genetic strategies. One key strategy is the development and deployment of resistant rice cultivars through breeding, focusing on incorporating durable resistance genes into high-yielding varieties. This method reduces the reliance on chemical fungicides, making it an environmentally sustainable option (Ashkani et al., 2015). In addition to breeding, timely application of fungicides can help control severe outbreaks, especially during favorable conditions for the pathogen. Proper field management, including balanced fertilizer application, especially nitrogen, optimal planting density, and maintaining good field drainage, also plays a crucial role in minimizing disease severity (Miah et al., 2017). Advanced tools like metabolomics and enzymatic profiling are increasingly being used to understand rice-plant interactions and develop targeted interventions against *M. oryzae* (Yang et al., 2024). Integrating these strategies into rice farming practices is vital to reducing yield losses and improving rice production sustainability.

P. oryzae is known for its genetic diversity and adaptability, making it challenging to control with conventional strategies. Consequently, understanding the physiological and biochemical defense mechanisms in rice has become a priority for developing sustainable management practices. Plants employ various defense strategies to counter pathogen attack, including structural barriers, secondary metabolites, and enzymatic

responses. Defense-related enzymes such as superoxide dismutase (SOD), phenylalanine ammonia-lyase (PAL), and chitinase (CHT) play critical roles in mitigating oxidative stress, biosynthesizing phenolic compounds, and degrading fungal cell walls, respectively (Prasannath, 2017). Additionally, lipoxygenase (LOX) and β-1,3-glucanase are involved in signalling and hydrolysing pathogen cell wall components. Reactive oxygen species (ROS), such as hydrogen peroxide and superoxide are also key mediators in plant-pathogen interactions, triggering localized cell death and systemic acquired resistance (Nisha et al., 2012).

Plants can rapidly produce reactive oxygen species (ROS) at the infection site. Reactive oxygen species (ROS) are created in plant cells under normal settings, but harsh environmental conditions might cause excessive production. These ROS degrade biomolecules and induce plant death. Plants use enzymatic and non-enzymatic antioxidants to regulate ROS generation and protect themselves from damage. Enzymatic antioxidant mechanisms like as superoxide dismutase and catalase reduce excess ROS, but non-enzymatic antioxidants such as vitamins and flavonoids protect against oxidative stress (Fahad et al., 2019).

The biochemical basis of rice resistance to *P. oryzae* has been explored in both susceptible and resistant varieties. Resistant rice cultivars exhibit enhanced activity of defense-related enzymes and higher accumulation of secondary metabolites like phenolics and flavonoids during pathogen attack (Chandrakanth et al., 2018; Yang et al., 2024). These metabolites not only strengthen plant cell walls but also exhibit direct antifungal properties, effectively inhibiting the growth and development of *P. oryzae* (Chakraborty and Islam, 2022).

Serious productivity losses due to blast infections have been reported in all rice-growing countries. However, detailed information on the defensive enzyme related to the physiological resistance of blast disease is limited from the perspective of Bangladesh. Defensive enzymatic response identification can be utilized in enzyme profiling, measurement of the activity of the defensive enzymes, gene expression analysis, and the development of resistant rice varieties through marker-assisted breeding or genetic engineering.

The objective of the study was to ascertain the defensive biochemical and enzyme responses of BRRI dhan29 and IR 64 linked to physiological resistance to *Pyricularia oryzae* stress.

Chapter II

Review of literature

Rice blast, caused by the fungal pathogen (*Pyricularia oryzae*), poses a significant threat to global food security due to its devastating impact on rice production. This disease affects rice crops in over 85 countries, making it one of the most important challenges in agriculture. The pathogen infects all above-ground parts of the rice plant, leading to yield losses that can exceed 50% under severe outbreaks (Asibi et al., 2019). Understanding the complex interactions between rice and *M. oryzae* is critical for developing effective management strategies. Recent advances in molecular biology have shed light on the dynamics of rice's resistance mechanisms, including the role of resistance (*R*) genes, signaling pathways, and defensive enzymes (Devanna et al., 2022). These studies emphasize the importance of integrating genetic resistance with other control methods to achieve sustainable management of rice blast, thereby contributing to global food security.

Global rice production faces various challenges, including pest and disease impacts, which significantly affect yield and sustainability. BRRI dhan29, a widely cultivated rice variety in Bangladesh, and IR 64, a globally recognized high-yielding mega-variety, are crucial to addressing food security. While BRRI dhan29 is known for its adaptability and productivity under subtropical conditions, IR 64's resilience and grain quality have made it a staple for millions globally (Mackill and Khush, 2018). However, diseases like rice blast, caused by *Magnaporthe oryzae*, pose substantial threats to these varieties, leading to reduced yields and economic losses. Rice blast management is critical as its outbreak not only diminishes productivity but also impacts the environment and profitability (Nalley et al., 2016; Simkhada and Thapa, 2022).

2.1 The role of defensive enzymes in plant stress tolerance and pathogen defense

Defensive enzymes play a pivotal role in enabling plants to withstand various biotic and abiotic stresses by enhancing their resilience and adaptability. These enzymes, such as superoxide dismutase, catalase, and peroxidases, are integral to the plant's enzymatic antioxidant defense mechanism, effectively neutralizing reactive oxygen species (ROS) generated during stress conditions (Rajput et al., 2021). Lipoxygenases, another key group of enzymes, are involved in lipid metabolism and signal transduction,

contributing to growth, development, and stress response by mediating processes such as jasmonic acid synthesis and membrane repair (Singh et al., 2022). Additionally, enzymes like phenylalanine ammonia-lyase and chitinases booster the plant's defense by reinforcing structural barriers and targeting pathogens directly (Chamandoosti, 2017). The interplay of these enzymes ensures a coordinated response to environmental challenges, highlighting their critical role in maintaining plant health and productivity under stress.

Superoxide dismutase (SOD) enzymes are vital components of the plant defense system, acting as the first line of defense against reactive oxygen species (ROS) by catalyzing the conversion of superoxide radicals into hydrogen peroxide and oxygen. Jiang et al. (2019) identified and analyzed the expression of SOD family genes in wheat, revealing their diverse roles in combating oxidative stress under different environmental conditions. Río et al. (2018) emphasized the significance of SODs in mitigating damage caused by abiotic stresses like drought, salinity, and temperature fluctuations, underscoring their protective role in maintaining cellular homeostasis. In rice, Li et al. (2019) demonstrated that the miR398b pathway modulates SOD activity, boosting hydrogen peroxide production to enhance resistance against rice blast (*Magnaporthe oryzae*). Similarly, Anushree et al. (2016 a) observed elevated SOD activity in rice genotypes resistant to rice blast, highlighting its role in reinforcing oxidative defense.

Phenylalanine ammonia-lyase (PAL) is a key enzyme in the phenylpropanoid pathway, playing a vital role in plant defense by synthesizing compounds like phytoalexins, lignin, and flavonoids, which enhance resistance to stress. Duan et al. (2014) reported that PAL activity significantly increased in rice roots infected by *Magnaporthe oryzae*, correlating with the accumulation of defense-related phytohormones and phytoalexins. Anushree et al. (2016 b) observed that rice genotypes resistant to rice blast exhibited elevated PAL activity compared to susceptible ones, highlighting its role in reinforcing structural barriers and activating biochemical defenses. Resistant rice genotypes demonstrated a stronger and quicker PAL-mediated response upon pathogen attack, indicating its role in early defense signaling. Keerthana et al. (2024) elaborated that resistant aromatic rice landraces exhibit upregulated PAL gene expression, emphasizing its involvement in developing systemic resistance.

Lipoxygenases (LOXs) are essential enzymes in plants that play a critical role in responding to stress conditions by mediating lipid peroxidation and producing bioactive metabolites. These enzymes are involved in the synthesis of signaling molecules like jasmonic acid and oxylipins, which regulate defense responses to both biotic and abiotic stresses, including pathogen attacks, drought, and salinity (Viswanath et al., 2020). LOXs contribute to cell membrane remodeling and stabilization during stress, ensuring cellular integrity under adverse conditions. Their metabolites, such as hydroperoxy fatty acids, act as signaling compounds that modulate gene expression linked to stress tolerance (Babenko et al., 2017). Lipoxygenase (LOX) activity highlights its significant role in plant-pathogen interactions and defense mechanisms. Debona et al. (2018) demonstrated that LOX-mediated pathways are activated in rice during infections by *Bipolaris oryzae*. These pathways enhance the plant's resistance, particularly when supported by strobilurin fungicides, which amplify oxidative defense responses and improve pathogen suppression. Similarly, Wennman et al. (2016) provided structural insights into manganese lipoxygenase from the rice blast fungus *Magnaporthe oryzae*. They revealed its role in the pathogen's lipid metabolism, which facilitates infection and host colonization. Together, these studies emphasize the dual roles of LOX: as a critical component of plant defense and a contributor to fungal pathogenicity, making it a key target for improving crop resistance strategies.

Chitinases play a crucial role in plant defense by degrading chitin, a major component of fungal cell walls, thereby impeding pathogen colonization and activating immune responses. Han et al. (2019) found that a chitinase from *Magnaporthe oryzae* interacts with a rice jacalin-related lectin, facilitating fungal colonization by manipulating host defense mechanisms. Yang et al. (2019) demonstrated that when *M. oryzae* chitinase MoChia1 is bound by a rice tetratricopeptide repeat protein, it allows free chitin to act as a damage-associated molecular pattern, triggering robust immune responses. Li et al. (2023) highlighted the protective role of chitinase from *Serendipita indica* in rice, where it conferred resistance to both blast and bakanae diseases by enhancing defense signaling pathways. Additionally, Negi et al. (2017) also reported the efficacy of chitinase-producing fluorescent *Pseudomonas* isolates in biologically controlling ragi blast disease, emphasizing their potential in sustainable agriculture.

during interactions between rice and the blast fungus *Magnaporthe oryzae*, where O_2^- acts as a signaling molecule to activate defense responses but also contributes to pathogen-induced oxidative stress. Marroquin-Guzman et al. (2017) revealed that, *M. oryzae* employs a nitro oxidative stress response to mitigate the impact of O_2^-, effectively suppressing rice innate immunity during blast disease. These findings underline the dual nature of superoxide in stress conditions, where its regulated presence is crucial for signalling and defense, yet its imbalance can be exploited by pathogens or lead to cellular damage.

Phenolic compounds play a crucial role in plants' defense against various stress conditions, acting as antioxidants, antimicrobial agents, and signaling molecules. Patzke and Schieber (2018) demonstrated the growth-inhibitory properties of phenolics like ferulic acid, which serve as natural pesticides against fungal pathogens such as *Botrytis cinerea*. Shalaby and Horwitz (2014) highlighted their role in mitigating oxidative stress during plant-fungal interactions by integrating defense and signaling pathways. Wallis and Galarneau (2020) revealed that phenolic induction is a consistent response in plant-microbe and plant-insect interactions, significantly enhancing resistance. Rashad et al. (2020) noted that these compounds effectively combat fungal and viral plant diseases by disrupting pathogen growth and infection processes. Gautam et al. (2020) emphasized their importance in sustainable agriculture by demonstrating their defensive functions against pathogenic microbes. Toan et al. (2017) observed that rice blast infection triggers an increase in phenolic and flavonoid content, enhancing antioxidant capacity to mitigate stress. Velmurugan et al. (2023) described the role of endophytic Firmicutes in inducing systemic resistance, partly through the production and accumulation of phenolic compounds, which strengthened rice defense against blast disease. These findings collectively underscore the multifaceted roles of phenolics in enhancing plant resilience under stress conditions.

Flavonoid compounds play a pivotal role in enhancing plant defense against various stress conditions, serving as antioxidants, phytoalexins, and signaling molecules. Chen et al. (2024) found that *Magnaporthe oryzae* infection activates rice defense against brown planthopper by modulating jasmonic acid pathways, which influence flavonoid biosynthesis. Toan et al. (2017) reported increased flavonoid accumulation in rice infected with *Pyricularia grisea*, boosting antioxidant capacity and aiding in stress

mitigation. Lahari et al. (2023) demonstrated that strigolactone-deficient rice accumulates jasmonates and flavonoid phytoalexins, significantly strengthening resistance to the blast fungus *Pyricularia oryzae*. Khalid et al. (2019) highlighted flavonoids' multifaceted roles in plant-environment interactions and as antimicrobial agents against pathogens. Shah and Smith. (2020) emphasized their dual role in responding to both biotic and abiotic stresses, along with fostering beneficial microbial associations. Hasegawa et al. (2014) analysed a flavonoid phytoalexin in rice, which accumulates in response to blast fungus infection, exhibits potent antifungal activity, and undergoes detoxification by the pathogen. These studies collectively underscore the integral role of flavonoids in bolstering plant resilience under stress conditions.

Antioxidant compounds are crucial in mitigating oxidative stress in plants, acting as protective agents against damage caused by reactive oxygen species (ROS) under various stress conditions. Bobrova et al. (2020) highlighted the importance of a balanced prooxidant-antioxidant system in maintaining plant immunity, suggesting that antioxidants play a central role in defense mechanisms. Derikvand et al. (2020) observed that antioxidant enzyme activity, such as catalase and peroxidase, fluctuates in common beans under bacterial infection, reflecting the adaptive response of plants to oxidative stress. Barreca (2020) reviewed the mechanisms of plant antioxidants, emphasizing their ability to scavenge ROS and regulate redox signalling, which is vital for stress tolerance. Aver'yanov et al. (2015) demonstrated that inhibiting antioxidant enzymes systemically reduced rice blast, revealing a nuanced interaction between oxidative stress and plant-pathogen dynamics. Together, these studies underscore the multifunctional role of antioxidant compounds in maintaining cellular integrity, enhancing stress tolerance, and supporting plant survival under adverse conditions.

Sugar compounds are vital for plant stress responses, acting as signalling molecules, energy sources, and modulators of defense mechanisms under biotic and abiotic stresses. Yao et al. (2022) highlighted the functional characterization of sweet sugar transporters, which not only regulate sugar flux but also contribute to plant resistance breeding by modulating pathogen interactions. Bezrutczyk et al. (2018) emphasized the role of sugar flux and signalling in plant-microbe interactions, demonstrating that sugars can influence pathogen growth and activate defense pathways. Similarly, Morkunas and Ratajczak (2014) reported that sugars act as crucial signalling molecules

in plant defense, regulating the expression of defense-related genes and enhancing resistance to fungal pathogens. These studies collectively illustrate the multifunctional role of sugar compounds in maintaining plant health, coordinating metabolic activities, and reinforcing defenses against environmental stresses.

Protein compounds play a pivotal role in plant responses to stress by regulating defense mechanisms and enhancing resilience against biotic and abiotic challenges. Lopes et al. (2023) elaborated on Pathogenesis-Related Protein 10 (PR-10), which contributes to plant immunity by acting as a signalling molecule and enzymatic player in defense pathways. Bundó and Coca (2017) demonstrated that OsCPK10, a calcium-dependent protein kinase, mediates rice drought tolerance and resistance to blast disease, highlighting its dual role in abiotic and biotic stress adaptation. Wu et al. (2021) identified the GroEL protein from *Rhodopseudomonas palustris* as a biocontrol agent enhancing rice resistance to blast disease by activating defense-related pathways. Collectively, these findings underscore the diverse roles of proteins in stress conditions, including signal transduction, enzymatic activity, and reinforcement of structural and functional defense, contributing to overall plant health and stress adaptation.

Chapter III

Materials and methods

Materials and methods for this study are presented in this chapter. This chapter describes the experimental site, climate, soil, experimental materials, experimental design, data collection and data analysis procedure.

3.1 Experimental site

The experiment was conducted at Plant Protection Laboratory and net house of Plant Breeding and Biotechnology laboratory of Agrotechnology Discipline, Khulna University.

3.2 Duration of the experiment

The experiment was conducted from January to March 2024.

3.3 Collection of seeds

Rice seeds of two genotypes (BRRI dhan29, IR 64) was collected from Bangladesh Rice Research Institute (BRRI), Gazipur. BRRI dhan29 is blast susceptible and IR 64 is blast resistant genotypes.

3.4 Collection of fungal isolates

A most virulent *P. oryzae* strain (RL-17) was used in this experiment. It was preserved in silica gel in a refrigerator at -86° C in the Plant Protection Laboratory of Agrotechnology Discipline, Khulna University.

3.5 Seed treatment

Seeds were treated with Vitavax-200 @ 0.3% (w/w). Seeds were mixed with Vitavax 200 in petridishes for one hour (Shahrin et al., 2016). The treated seeds were then plated on water-soaked filter papers in petridishes for germination.

3.6 Soil and Pot Preparation

The soil filled in the pot was collected from the non-saline zone of the Khulna division. The physical and chemical properties of collected soil were clay loam textured, pH-6.6, EC- 1.02, Soil organic matter- 1.87%, available N- 1140.5 ppm, available P- 15.04 $\mu g g^{-1}$, available S- 176.8 $\mu g g^{-1}$ with an inherent exchangeable K, Ca, and Mg content at 0.65 Cmol kg^{-1}, 16.5 Cmol kg^{-1}, and 6.5 Cmol kg^{-1} soil, respectively. After 7 days of sun drying, the soil was gently crushed with a hammer to break up large particles. After

removing undesired elements such as dried roots, grass, and stones, the soil was properly ground. 15 kgs of soil were placed in each plastic pot with compost and sand in a ratio of 2:1:1 and the pot size was (30×20×14) cm. Before adding soil, plastic pots were rinsed with tap water and dried in the sun. The experiment was designed using the factorial completely randomized design (CRD) with three replications. Each pot was counted as one replication. Each pot received equal amounts of fertilizer (urea, TSP, MoP, and gypsum) at rates of 1.3, 0.5, 0.6, and 0.4 g pot^{-1} for nitrogen, phosphorus, potassium, and sulfur (Rahman et al.,2007). Before 2 days of transplantation, the first split dose of urea and full doses of all other fertilizers were manually mixed into the soil. The second split dose of urea was applied after 35 days (maximum tillering stage) of planting.

3.7 Germination and transplanting of seeds

The treated seeds were placed on soaked filter paper on petridish for germination and later incubated at 28°C in the dark for 5 days until they germinate. Afterwards, 25 seedlings were sown into each plastic pots. Before transplanting the soil was watered thoroughly to ensure it is evenly moist but not waterlogged. After transplanting the seeds were lightly covered with a thin layer of soil. The pots were watered gently using a sprinkler to avoid displacing the seeds.

3.8 Culture of *P. oryzae*

Initially, the *P. oryzae* strain was cultured for 7 days at 23°C on a Potato Dextrose Agar (PDA) culture medium. A mycelial lawn with a diameter of 2 mm was cut using a borer, transferred to an oat agar medium, and cultured for 14 days in dark conditions at 23°C. After 14 days of transferring into oat agar media, mycelial growth was scrapped by using sterilized toothbrushes. Scraped Petri dishes were covered with transparent plastic with proper ventilation for air passing and they were kept under continuous light condition at 24°C for 72 hours. *P. oryzae* sporulation was observed under a microscope. After confirming sporulation, distilled water was added to the sporulated plates, and the plates were rubbed gently with a painting brush. Spore suspensions were filtered through a gauze. Spore suspensions were utilized as inoculants with a 3×10^5 mL^{-1} concentration. Tween 20 was added to this spore suspension so that spores could easily be attached to the leaf.

- Total antioxidant (μg ml^{-1}).
- Total soluble sugar (μg g^{-1}).
- Total soluble protein (mg g^{-1}).

3.13.1 Superoxide dismutase (SOD)

Superoxide dismutase (SOD) activity was measured by inhibiting the photoreduction of nitroblue tetrazolium (NBT) by the SOD enzyme by following the procedure of Del Longo et al. (1993) with some modification. 0.5gm fresh leaf sample was taken and 100 mM potassium phosphate buffer was (pH 7) added. Then the mixture was centrifuged at 5000 rpm for 10 min at 4°c and the supernatant was collected for measurement of enzymatic activity. The reaction mixture included 40 mM sodium phosphate buffer and the pH was 7.8, 13 mM methionine, 75 μM NBT, 2 μM riboflavin, and 100 μl crude extract in a final volume of 3.0 ml. A control reaction was conducted without crude extract. 1 ml enzyme was taken in test tube and 2.5 ml methionine,0.3 ml riboflavin, 0.1 ml NBT were added respectively. The SOD reaction was exposed to white light for 30 minutes at room temperature and absorbance was recorded at 560 nm using a spectrophotometer. The enzyme that inhibited NBT photochemical degradation by 50% was considered to have one unit of SOD activity.

$$SOD = \frac{(Abs\ blank - Abs\ sample)}{Abs\ blank} \times \frac{Volume\ of\ reaction\ mixture}{time \times 0.01}$$

3.13.2 Phenylalanine ammonia-lyase (PAL)

Phenylalanine ammonia-lyase (PAL) was tested according to Shamim et al. (2018). The study involved homogenizing 1g leaves samples into a solution containing 0.1 M sodium borate buffer, pH 7.0, 2-mercaptoethanol, and insoluble poly vinyl pyrrolidone (PVPP). The extract was filtered and centrifuged at 16,000 x g for 15 minutes. The supernatant was used as an enzyme source. PAL activity was determined by calculating the rate of conversion of L phenylalanine to transcinnamic acid at 290 nm. A sample of 0.4 ml enzyme extract was incubated with 0.5 ml of 0.1 M borate buffer (pH 8.8) with 0.5 ml of 12 mM L-phenylalanine in the same buffer for 30 minutes at 30°C. The quantity of trans-cinnamic acid synthesized was determined. The enzyme activity was

μ moles of trans-cinnamic acid released per min per mg proteins (μM min^{-1} mg^{-1} protein)

3.13.3 Lipoxygenase (LOX)

The enzyme activity was measured spectrophotometrically following the procedure of Ben Aziz et al. (1970) and Aluri (2015). 1gm of leaf sample were homogenized in 100 mM potassium phosphate buffer (pH 6.3) containing 2 mM ascorbic acid, 2 mM EDTA, and 5 mM sodium metabisulphite. The homogenized mixture was passed through two layers of cheesecloth and the extract was centrifuged at 10,000 rpm for 20 min at 4°C. The clear supernatant was collected obtained and used as crude enzyme for estimation of lipoxygenase. The crude enzyme extract was fractionated at 45% ammonium sulphate. The enzyme extract was centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was removed and the resulting pellet was resuspended in 50 mM phosphate buffer (pH 6.3), with the contents dialyzed against the same buffer. At room temperature, 40 μg rice leaf LOX was added to a buffer of 100 mM phosphate at pH 6.3. The reaction started with 250 μM AA and lasted 1 minute. The activity of the enzyme was measured at 235 nm using a UV-Vis spectrophotometer, and it was defined as the number of moles of hydroperoxide generated per minute per mg of protein.

$$\text{Enzyme Activity} = \frac{\text{Volume of reaction mixture} \times \text{absorbance difference}}{\varepsilon \times \text{volume of enzyme}}$$

Where, $\varepsilon = 27{,}500$.

3.13.4 Chitinase

Chitinase was measured by using the protocol of Byrne et al. (2001). At first, 4 g leaf samples were homogenized in a grinder, and the homogenate was diluted 4:10 w/v with 10 ml of 50 mM sodium buffer pH 5.0. Approximately 1.5 ml of the resulting solution was transferred to an Eppendorf microcentrifuge tube and centrifuged at 10,000 rpm for 10 min. Initially, a 1:10 dilution was used with 50 mM sodium acetate buffer pH 5.0 in the assay. 0.2 ml extract was incubated at 25°C in an Eppendorf microcentrifuge tube with 0.2 ml of CM-Chitin-RBV and 0.4 ml of 50 mM sodium acetate buffer pH 5.0 for 0, 10, 20, 30, 40, 50, and 60 min. The reaction was stopped by the addition of 0.2 ml of 12 M HCl, and the tube was cooled on ice for 10 min. Then the tubes were centrifuged at 10000 g for 5 min, and the supernatant was transferred to a 1 cm path

length reduced volume (1 ml) disposable plastic cuvette. The absorbance was measured at 550 nm.

3.13.5 β-1,3-glucanase

The activity of β-1,3-glucanase enzyme was estimated by following the method described by Ippolito et al. (2000). Chilled leaves (0.1 g) were ground in a mortar and pestle with 1 ml of 0.05 M sodium acetate buffer, pH 5.0 and centrifuged at 13,000 rpm for 10 min. Supernatant was stored at -18◦C and residues were discarded. For determining enzyme activity, 100 µl of crude enzyme solution was taken and added with an equal volume of 0.05 mol l^{-1} sodium acetate solution (containing 5 mg ml^{-1} laminarin, pH 5.0). The mixture was incubated in a water bath and maintained at 37°C for 1 h. Afterward, 600 µl of 3,5-dinitrosalicylic acid (DNS) was added, and the resulting mixture was heated in a water bath for 5 min at 100°C. The mixture was cooled and diluted with distilled water. The reducing sugar content was determined by using a colorimeter at 540 nm. The one unit of enzymatic activity was expressed as a gram of tissue generating 1 mg of reducing sugar per hour.

3.13.6 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) was assayed according to Velikova et al. (2000). At first 500 mg leaf tissues (500 mg) were homogenized in an ice bath with 5 ml of 0.1% TCA (trichloroacetic acid) solution. The mixture was then centrifuged at 12,000 rpm for 15 minutes, and 0.5 ml of the resulting supernatant was combined with 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The absorbance of the supernatant was measured at 390 nm, and H_2O_2 content was determined using a standard curve. It was measured as micromoles per kilogram of fresh weight.

3.13.7 Superoxide (O_2^-)

Superoxide was measured according to the protocol of Mir (2014-2015) with some modification. At first, 5 g of the sample was taken, and the sample was ground finely with liquid nitrogen. 4.5 ml of distilled water was added, and after 10 min, 5 ml of distilled water was added and centrifuged at 4000 rpm for 15 min. Pyrogallol stock solution was made by adding 56.7 mg pyrogallol, 10 mM HCl, and 100 ml distilled water. An alkaline mixture was prepared by adding 10 ml of 0.25 M bicarbonate solution, 10 ml of water, and 5 ml of 0.1 M sodium hydroxide solution. Then 3.5 ml

aliquots were taken, and 0.5 ml of alkaline mixture was added and placed in a dark condition for 40 min. Then the absorbance was taken at 415 nm.

3.13.8 Total phenolic content

Total phenolic content (TPC) was estimated according to the method of Slinkard and Singleton (1977). The plant sample was prepared by dissolving 4.3 mg in 10 ml methanol. In a test tube, 300 µl of the solution, 1 ml of methanol, 3.16 ml of distilled water, and 200 µL of Folin-Ciocalteu reagent were added. After an 8-minute incubation at ambient temperature, 600 µl of 10% sodium carbonate solution was added to the test tube, which was then covered with aluminum foil and incubated in a hot water bath at 40°C for 30 minutes. A blank was created by following the same technique but replacing the plant extract with an equal volume of methanol. A UV-visible spectrophotometer was used to determine the sample's absorbance at 765 nm. The same process was followed to obtain the gallic acid standard curve. The total phenolic content was measured as µg of gallic acid equivalents (GAE) per ml, which was calculated using the formula $y = 0.0425\ x - 0.0247$, where y is the absorbance at 765 nm and x is the amount of gallic acid equivalent (µg ml^{-1}).

3.13.9 Total flavonoid content

Total flavonoid was measured by the protocol of Ahmed et al. (2014). Each extract solution was made by sonicating 3 mg of the sample in 10 ml of methanol for 10 minutes. In a test tube, 300 µl of extract (0.3 mg ml^{-1} in methanol) was mixed with 3.4 mL of 30% aqueous methanol to create a clear solution. After that, 150 µl of a 0.5 M aqueous sodium nitrite solution and 150 µL of a 0.3 M aluminum chloride solution were added. After 5 minutes, 1 ml sodium hydroxide solution (1 M) was added and thoroughly mixed before measuring the absorbance at 506 nm on a UV-visible spectrophotometer against a blank generated using the same process but replacing the plant extract with an equivalent volume of methanol. A rutin calibration curve was obtained for concentrations ranging from 75 mg l^{-1} to 750 mg l^{-1}. The total flavonoid content of leaf extract was expressed as µg of rutin equivalents (RE) per ml, calculated using the formula $y = 0.0236\ x + 0.0348$, where y is the absorbance at 506 nm and x is the amount of rutin equivalent (µg ml^{-1}).

3.13.10 Total antioxidant capacity

Total antioxidant capacity (TAC) was measured using the phosphomolybdate method, which was described by Ahmed et al. (2015). 250 µg of plant extract was dissolved in 1 ml of methanol and sonicated for 5 minutes to ensure a uniform mixture. As a standard, ascorbic acid was employed. A stock solution of ascorbic acid at a concentration of 5000 mg l^{-1} was made using distilled water, from which subsequent dilutions were prepared, ranging between 25 mg l^{-1} and 500 mg l^{-1}. In a test tube, 300 µl of the plant extract was combined with 3 ml of phosphomolybdate reagent, which consists of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The test tube was then covered with aluminium foil and incubated at 95 °C for 90 minutes. After reaching room temperature, the absorbance of the mixture was measured at 765 nm. A blank was also prepared using the same procedure but replacing the plant sample with an equivalent volume of methanol. The antioxidant capacity was expressed in terms of µg of ascorbic acid equivalents (AAE) per ml.

3.13.11 Total soluble sugar

The anthrone method was used to determine sugar according to Plummer (1990). Then 6 N HCl were prepared, and 5 ml HCl were taken in test tubes. Test tubes were placed in a water bath and heated for 1 hour at 80 °C. 9 ml of distilled water was mixed with 1 ml of extracted sample. 1 ml of the diluted sample was transferred to another test tube. To each tube, add 5 ml of the anthrone reagent which was mixed well by vortexing. The tubes were cooled, and absorbance was taken at 620 nm. A blank was prepared with 1000 µl distilled water and 5 ml anthrone reagent. Standard samples with known glucose volumes (50, 100, 200, 300, 400, 500, 750, 1000 µl) along with corresponding DH_2O volumes and anthrone reagent were prepared. A standard curve was made of absorbance vs. µg glucose. The amount of glucose in the unknown sample was determined by plotting a standard curve of A620 on the Y-axis and µg of glucose on the X-axis.

3.13.12 Total soluble protein

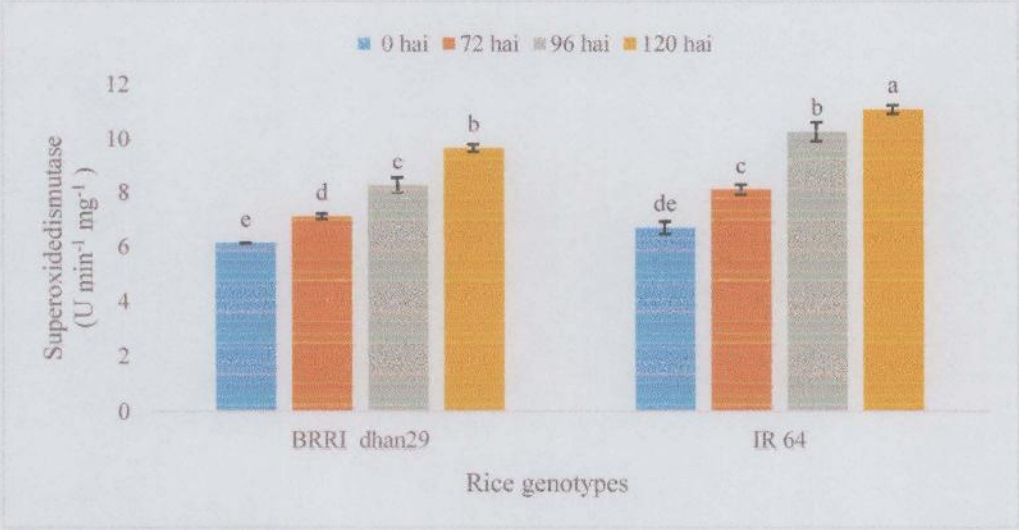
Protein was estimated by the Bradford method described by Bradford (1976). Protein extraction buffer was made with Tris HCl (pH 8.1) 10 mM, EDTA (pH 8.0) 10 mM, β-mercaptoethanol 5 mM, and PMSF 0.1 mg ml^{-1}. At first, a 1 g sample was weighed, and 1 ml of protein extraction buffer was added per gram of leaf in a cold mortar and pestle. The tissue was grinded in liquid nitrogen until a thick paste was produced. The paste was collected and placed in a 1.5 ml microcentrifuge tube and centrifuged for 20 minutes at 12000 rpm at 4°C. The supernatant was transferred to another 1.5 ml microcentrifuge tube, and 100 µl of the supernatant was taken in a tube for quantification of the extracted protein by the Bradford method. Two different concentrations of the extract (20 µl and 5 µl) were taken. 5 ml of dye reagent was added and mixed well. At the same time, a set of standards containing 5, 10, 20, 30, 40, 50, and 100 µl of Bovine Serum Albumin (BSA 2.0 mg ml^{-1} stock in extraction buffer) was prepared in separate tubes. Extraction buffer was added to each tube to bring the volume to 100 µl. To these tubes, 5 ml of dye reagent was added and mixed well by vertexing. After 5 minutes, the absorbance was taken at 595 nm (OD595) against a reagent blank (100 µl of extraction buffer with 1 ml of dye reagent). A standard curve was prepared of absorbance versus micrograms of protein, and the slope y/x from the standard curve was determined, which gave the A595 per unit of protein (µg).

Chapter IV

Results

4.1 Superoxide dismutase (SOD)

Superoxide Dismutase (SOD) activity in two rice cultivars, BRRI dhan29 and IR 64, under *pyricularia oryzae* stress is presented in Figure 1. In BRRI dhan29, SOD activity in the control condition was the lowest (approximately 6.13 U min^{-1} mg^{-1}). After 72 hours of inoculation, there was a significant increase (7.11 U min^{-1} mg^{-1}), followed by further increases at 96 hours (approximately 8.26 U min^{-1} mg^{-1}) and peaking at 120 hours (9.64 U min^{-1} mg^{-1}). In IR 64, the control condition also exhibited lower SOD activity (6.69 U min^{-1} mg^{-1}), which significantly increased after 72 hours (8.1 U min^{-1} mg^{-1}). Further increases were observed at 96 hours (10.25 U min^{-1} mg^{-1}), with the highest activity recorded at 120 hours (11.06 U min^{-1} mg^{-1}). Between the two varieties, IR 64 exhibited consistently higher SOD activity across all time points compared to BRRI dhan29. This indicates a potentially stronger oxidative stress response in IR 64.

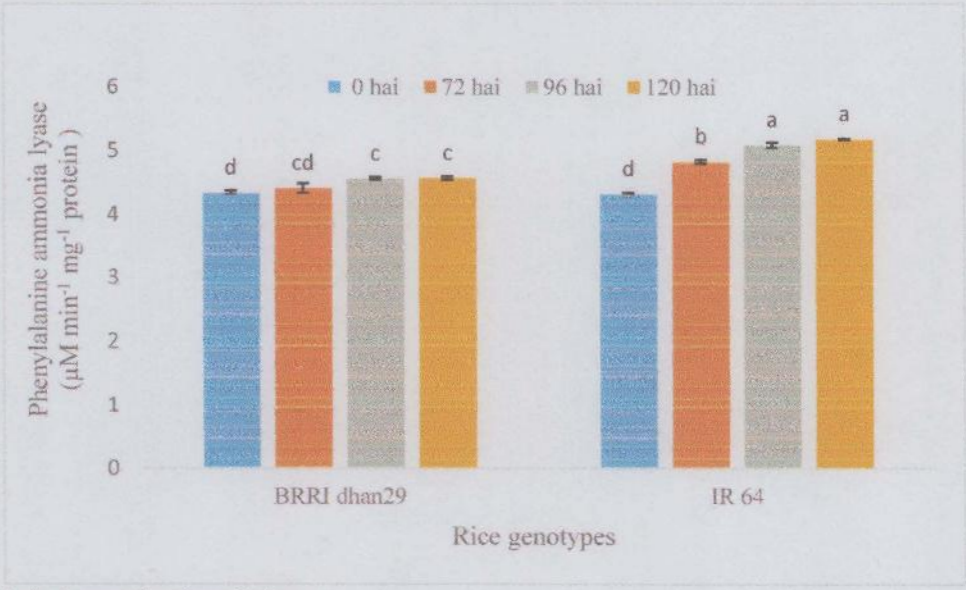


hai= hours after inoculation

Figure 1 .Activity of superoxide dismutage (SOD) of BRRI dhan29 and IR 64 against *P. oryzae* stress. All values represent the mean ± standard error (n= 3) at P <0.05 probability according to Tukey HSD All-Pairwise Comparisons Test.

4.2 Phenylalanine ammonia lyase (PAL)

The activity of Phenylalanine Ammonia-Lyase (PAL), measured in ($\mu Mmin^{-1}mg^{-1}$protein) of two rice varieties, BRRI dhan29 and IR 64, under control conditions and 72, 96, and 120 hours after inoculation is illustrated in Figure 2. In the case of BRRI dhan29, PAL activity remains relatively constant across the control, 72, 96, and 120 hours after inoculation, with minor variations. The values for all treatments are within a similar range (4.34 to 4.57). The statistical data indicates that there was no notable rise in PAL activity over time. In the case of IR 64, PAL activity showed a more pronounced increase across treatments, with values ranging from 4.32 to 5.18. The data indicate that PAL activity in IR 64 increased significantly with increasing time after inoculation, whereas BRRI dhan29 maintains a consistent PAL activity level.

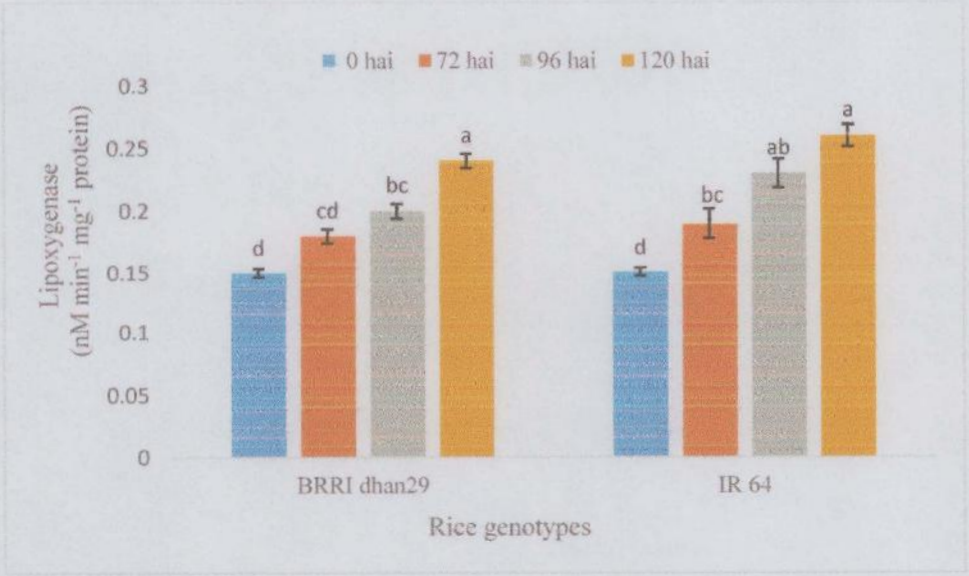


hai= hours after inoculation

Figure 2. Activity of phenylalanine ammonia lyase (PAL) of BRRI dhan29 and IR 64 against *P. oryzae* stress. All values represent the mean ± standard error (n= 3) at $P < 0.05$ probability according to Tukey HSD All-Pairwise Comparisons Test.

4.3 Lipoxygenase (LOX)

The lipoxygenase (LOX) activity of two rice cultivars, BRRI dhan29 and IR 64, is shown in Figure 3. The LOX activity of BRRI dhan29 gradually increased with duration after inoculation, showing a clear trend from the Control to 120 hours. The Control group (no inoculation) had the lowest LOX activity (0.15 nM min^{-1} mg^{-1} protein) and after 120 hours, the highest activity (0.26 nM min^{-1} mg^{-1} protein) was observed. Statistical groupings indicate significant differences among treatment durations. Similar to BRRI dhan29, LOX activity of IR 64 increased with treatment duration. Statistical analysis indicates significant differences between treatment times, indicating a time-dependent increase in LOX activity for both BRRI dhan29 and IR 64 but there was no significant difference between these two genotypes.

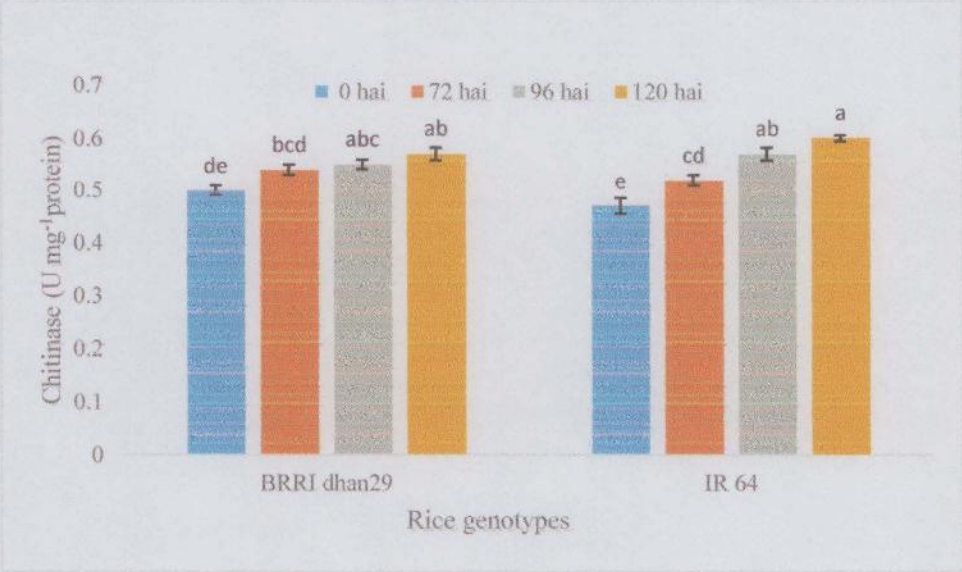


hai= hours after inoculation

Figure 3 . Activity of lipoxygenase (LOX) of BRRI dhan29 and IR 64 against *P. oryzae* stress. All values represent the mean ± standard error (n= 3) at $P < 0.05$ probability according to Tukey HSD All-Pairwise Comparisons Test.

4.4 Chitinase (CHT)

The chitinase enzyme activity, expressed in units (U) per mg of protein, in two rice cultivars, BRRI dhan29 and IR 64, under control condition and different treatment durations after inoculation is shown in Figure 4. Chitinase activity of BRRI dhan29 gradually increased from the Control to the 120 hours. Statistical groupings highlight significant differences among different times, where the 120 hour and 96 hours groups showing higher chitinase activity than the Control. Similar to BRRI dhan29, chitinase activity of IR 64 increased with treatment duration. In case of IR 64, control had the lowest chitinase activity (0.47) and after 120 hours, the highest activity (0.6) was observed indicating a statistically significant difference from the earlier time points. Chitinase activity showed a time-dependent increase in both rice cultivars, but statistically there was very slight difference between two genotypes.

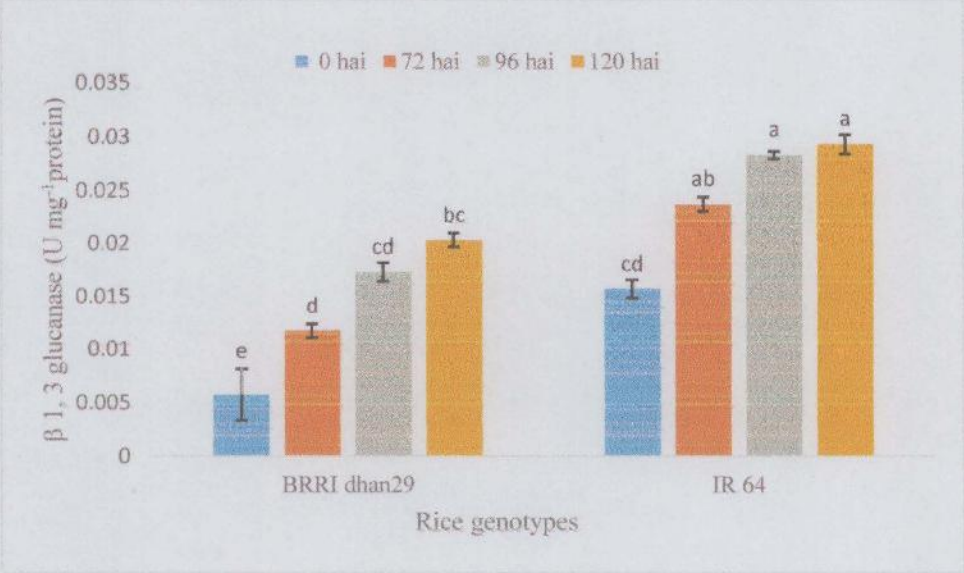


hai= hours after inoculation

Figure 4. Activity of chitinase (CHT) of BRRI dhan29 and IR 64 against *P. oryzae* stress. All values represent the mean ± standard error (n= 3) at $P < 0.05$ probability according to Tukey HSD All-Pairwise Comparisons Test.

4.5 β 1, 3 glucanase

The activity of β 1, 3 glucanase, measured in units per mg of protein in two rice cultivars, BRRI dhan29 and IR 64, at control conditions and different time intervals after inoculation is illustrated in Figure 5. The graph suggests that β 1, 3 glucanase concentration increased over time in both rice varieties but IR 64 showing a more significant increase at every treatment compared to BRRI dhan29. The highest β 1, 3 glucanase levels were observed at 120 hours for both varieties. The increased level of β 1, 3 glucanase was more noticable in IR 64. In case of IR 64 the value of control was 0.0157 that increased to 0.0293 in 120 hours whereas the control value of BRRIdhan29 was 0.0057 that increased to 0.0203 in 120 hours. There was a statistically significant difference between two cultivars. The level of β 1, 3 glucanase was higher in IR 64 than BRRI dhan29.



hai= hours after inoculation

Figure 5. Activity of β 1, 3 glucanase of BRRI dhan29 and IR 64 against *P. oryzae* stress. All values represent the mean ± standard error (n= 3) at $P < 0.05$ probability according to Tukey HSD All-Pairwise Comparisons Test.

4.6 Hydrogen peroxide (H_2O_2)

The concentrations of hydrogen peroxide (H_2O_2) in two distinct types of rice cultivars (BRRI dhan29 and IR 64) at control condition and different time intervals after inoculation are presented in Table 1. Initially, the level of H_2O_2 of BRRI dhan29 was relatively minimal but there was a significant rise in 72 hours after inoculation. Levels still increased at 96 hours and the peak concentration of H_2O_2 was seen at 120 hours. The initial H_2O_2 level of IR 64 was similar as BRRI dhan29. There was a gradual increase in H_2O_2 levels at 72 and 96 hours after inoculation. The maximum H_2O_2 concentration was reached at 120 hours, similar to BRRI dhan29. Initially the level was similar for two cultivars but the at 96 hour and 120hour the level of H_2O_2 was higher in BRRI dhan29. Statistically there was a significant difference between two genotypes after 96 and 120 hours after inoculation.

Table 1. Hydrogen peroxide and superoxide concentrations of BRRI dhan29 and IR 64 against *P. Oryzae* stress.

Rice genotypes	Treatment (hai)	Hydrogen peroxide (μmole kg^{-1} FW)	Superoxide (mmole kg^{-1} FW)
BRRI dhan29	0	77.73 e	0.97 f
	72	121.91 d	1.17 e
	96	169.03 b	1.44 b
	120	196.72 a	1.55 a
IR 64	0	86.72 e	1.21 de
	72	122.21 d	1.29 cd
	96	148.78 c	1.38 bc
	120	179.21 b	1.46 ab
CV (%)		3.89	2.50

hai = hours after inoculation
Values with different letters are significantly differed at 5% level of probability according to Tukey's HSD All-Pairwise Comparisons Test

4.7 Superoxide

The concentration of superoxide (SO) in two rice varieties, BRRI dhan29 and IR 64, under different treatment durations, measured in millimoles per kilogram of fresh weight (mmole kg^{-1} FW) is shown in Table 1. Before inoculation the SO concentration of BRRI dhan29 was very low. The concentration increased progressively after inoculation, reaching the highest level at 120 hours, indicating a significant increase compared to other durations. At 72 and 96 hours after inoculation BRRI dhan29 showed intermediate concentrations respectively. The control group in IR 64 also had a lower SO concentration compared to the treated groups. The concentration increased with longer treatment durations but it was not as evident as BRRI dhan29. BRRI dhan29 showed more pronounced response in SO concentration with extended treatment durations compared to IR 64 but statistically there was very slight difference between these two genotypes.

4.8 Total phenol

The level of phenol in BRRI dhan29 and IR 64 rice before inoculation and after inoculation is shown in Table 2. The phenol concentration was quantified in micrograms per millilitre (μg ml^{-1}), with distinct colours assigned to each treatment. The phenol concentration in the control group was lower than in the other groups. The level of phenol raised slightly as time increased although this increase was not very significant when compared to shorter times. In IR 64, the control group had the lowest phenol concentration and the concentration notably rose as treatment time increased. The concentrations at 72 hours and 96 hours showed a moderate rise in phenol production as time progressed. IR 64 showed a more pronounced and significant increase in phenol concentration with prolonged treatment durations compared to BRRI dhan29, which exhibits a more stable phenol response.

4.9 Total flavonoids

The concentration of flavonoids measured in micrograms per millilitre (μg ml^{-1}) is shown in Table 2. For BRRI dhan29, flavonoid concentration increased gradually with time after inoculation, reaching the highest level at 120 hours, indicating a significant increase from the control group. In contrast, IR 64 showed a more noticeable increase, after 96 hour and 120 hours after inoculation and reaching the same maximum concentration significantly higher than the control. Initially, the flavonoid concentration was 34.96 which increased to 46.29 after 120 hours. This trend suggests

that IR 64 accumulates flavonoids more rapidly in response to prolonged treatment, potentially indicating a stronger flavonoid-mediated stress response than BRRI dhan29.

Table 2. Activity of total phenol, total flavonoids, total antioxidant, total soluble sugar, total soluble protein of BRRI dhan29 and IR 64 against *P. oryzae* stress.

Rice genotypes	Treatment (hai)	Total phenol ($\mu g\ ml^{-1}$)	Total flavonoids ($\mu g\ ml^{-1}$)	Total antioxidant ($\mu g\ ml^{-1}$)	Total soluble sugar ($mg\ g^{-1}$)	Total soluble protein ($\mu g\ g^{-1}$)
BRRI dhan29	0	12.63 ef	38.44 d	300.08 d	3.13 f	39.7 d
	72	13.23 de	41.27 c	313.41 d	3.34 e	55.7 cd
	96	13.49 cd	42.38 bc	337.69 c	3.29 e	63.82 bc
	120	13.93 bcd	43.42 b	344.87 c	3.31 e	68.76 bc
IR 64	0	11.98 f	34.96 e	317.38 d	4 d	56.21 cd
	72	14.06 bc	39.67 d	351.14 c	4.18 c	76.57 b
	96	14.37 b	45.28 a	401.6 b	4.44 b	107.88 a
	120	15.5 a	46.29 a	443.21 a	4.75 a	124.44 a
CV (%)		1.84	1.07	1.87	1.27	8.22

hai = hours after inoculation
Values with different letters are significantly differed at 5% level of probability according to Tukey's HSD All-Pairwise Comparisons Test

4.10 Total antioxidant

The concentration of total antioxidant compounds in two rice varieties measured in micrograms per millilitre ($\mu g\ ml^{-1}$) is shown in Table 2. In BRRI dhan29, antioxidant levels were mostly steady and showed a small increase. In contrast, IR 64 showed a more significant increase in antioxidant concentration with prolonged treatment, reaching the highest level at 120 hours. This pattern suggests that IR 64 has a significant antioxidant response to extended treatment durations, potentially indicating greater resilience or adaptation to stress compared to BRRI dhan29.

4.11 Total soluble protein

The concentration of protein in different treatment durations in micrograms per gram ($\mu g\ g^{-1}$) is shown in Table 2. Initially, the concentration was low for both varieties. The concentration of BRRI dhan29 rose progressively with the treatment duration, peaking

at 120 hours, though the increase between 96 and 120 hours is minor. There was a notable increase in protein concentration of IR 64 as the treatment progressed, with a more substantial rise between the control and 72 hours compared to BRRI dhan29. The table indicates that both rice varieties respond to the treatment with an increase in protein concentration, with IR 64 showing a more pronounced response.

4.12 Total soluble sugar

The total soluble sugar concentration in two rice varieties, under control conditions and after 72, 96, and 120 hours of inoculation is shown in Table 2. The sugar levels of BRRI dhan29 remain relatively stable across all time points, with values ranging between 3.13 to 3.3 (mg g^{-1}). There was no noticeable difference visualized at the various time intervals. In IR 64 sugar content increased progressively from the Control to the 120-hour with the value ranging between 4 to 4.75mg g^{-1}. A statistically significant difference was noticed between all treatments and between two varieties. The IR 64 variety shows a significant increase in sugar concentration over time, whereas BRRI dhan29 maintains a relatively constant sugar level across all treatments.

Chapter V

Discussion

Blast is the most important and affective disease in rice crop worldwide. Blast disease of rice was observed in 10 agro-ecological zones (AEZs) of Bangladesh Hossain et al. (2017). Yield losses due to this disease can range from 10% to 30% under moderate infection conditions but may escalate to as high as 80% or complete crop failure in severe outbreaks Asibi et al. (2019). To manage the disease, it is vital to identify potential sources of resistance. New genotypes for host resistance are the greatest method to control blast disease. For this reason, biochemical profiling is necessary. The study was conducted to profile the defense-related enzymes and chemicals.

This study found a gradual increase in superoxide dismutase (SOD) activity in rice cultivars after *Pyricularia oryzae* inoculation, demonstrating a strong antioxidant defense against pathogen infections. The consistently higher SOD activity in IR 64 compared to BRRI dhan29 (Figure 1) across all time points suggests a more robust oxidative stress response in IR 64. These findings reflect previous research that has highlighted the importance of SOD in reducing oxidative stress during biotic stress situations. Li et al. (2019) demonstrated the involvement of *Osa-miR398b* in boosting H_2O_2 production and conferring enhanced blast resistance through the regulation of SOD expression The trend of increasing SOD activity over the time points (72 to 120 hours post-inoculation) also aligns with findings by Neng et al. (2017), who reported that antioxidant enzyme activities, including SOD, are progressively induced in rice with enhanced resistance to rice blast.

The phenylalanine ammonia-lyase (PAL) activity in BRRI dhan 29 and IR 64 after *Pyricularia oryzae* infection reveals their distinct defense mechanisms. BRRI dhan29 showed stable activity, while IR 64 showed a significant increase, indicating stronger enzyme induction (Figure 2). This progressive rise indicates an active and robust defense mechanism in response to pathogen attack. The findings align with Keerthana et al. (2024), who reported enhanced PAL activity and its correlation with disease resistance in blast-resistant rice varieties. The increased PAL activity in IR 64 suggests that this cultivar effectively upregulates the phenylpropanoid pathway to strengthen its defense through the accumulation of protective compounds like phytoalexins and

lignin. Duan et al. (2014) also highlighted the role of phytoalexins, regulated by enzymes like PAL, in restricting pathogen growth and spread. The lack of a significant increase in PAL activity suggests that BRRI dhan29 may have a weaker capacity to induce these critical defense compounds during infection.

The observed increase in lipoxygenase (LOX) activity in both BRRI dhan29 and IR 64 following *Pyricularia oryzae* inoculation highlights the enzyme's role in plant defense mechanisms. The significant time-dependent rise in LOX activity reflects its involvement in signalling pathways and the production of defense-related compounds (Figure 3). Singh et al. (2022) emphasized the role of LOX in stress signalling, particularly in the production of JA, a signalling molecule essential for activating defense genes during pathogen attack. Wennman et al. (2016) also investigated the structural properties of fungal manganese LOX, which interact with host plants. These interactions frequently activate LOX activity in the host as a counter-defense response.

In this investigation, BRRI dhan29 and IR 64 demonstrated an identical increase of chitinase activity showed in (Figure 4), although the degree and timing of activity differed due to genotypic differences explored by Anushree et al. (2016 b) and Kalboush (2019). They demonstrated similar time-dependent increases in chitinase activity in rice genotypes challenged with fungal pathogens. Their studies emphasized that higher chitinase activity correlates with enhanced resistance.

In this study, both rice cultivars showed enhanced β-1,3-glucanase activity over time but IR 64 showed an increased response than BRRI dhan29 (Figure 5), indicating the enzyme's crucial function in plant defense mechanisms against *Pyricularia oryzae*. β-1,3-glucanase destroys β-1,3-glucans in fungal cell walls, reducing pathogen proliferation and increasing resistance. Wang et al. (2021) described a novel β-1,3-glucanase (*Gns6*) in rice with potent antifungal activity against *M. oryzae*. The observed increase in β-1,3-glucanase activity in IR 64 aligns with such findings, indicating that the cultivar mobilizes this enzyme as a frontline defense mechanism to degrade fungal cell walls and inhibit pathogen progression.

H_2O_2 serves as a critical reactive oxygen species (ROS) that mediates defense signaling and pathogen-induced cell wall fortification. BRRI dhan29 displayed a rapid and pronounced accumulation of H_2O_2, with levels significantly rising at 72 hours post-inoculation and peaking at 120 hours (Table 1) This sharp increase suggests an

aggressive oxidative burst, a key component of plant innate immunity. Gupta et al. (2021) reported distinct H_2O_2 dynamics in plant-pathogen interactions, emphasizing its role in activating downstream defense mechanisms, including the hypersensitive response and oxidative burst. Aver'yanov et al. (2015) also highlighted that H_2O_2 enhances defense signaling, while excessive accumulation may lead to oxidative damage.

The higher and more pronounced SO accumulation in BRRI dhan 29 compared to IR 64 (Table 1) suggests a stress-induced overproduction of reactive oxygen species (ROS) in the susceptible variety, potentially due to an inefficient ROS-scavenging system. Aver'yanov et al. (2015) emphasized the dual role of ROS, particularly superoxide, in plant-pathogen interactions. While ROS accumulation can trigger defense mechanisms like the hypersensitive response (HR) and cell wall fortification, excessive ROS, as seen in BRRI dhan29, may cause oxidative damage and compromise cellular integrity, making the plant more vulnerable to the pathogen.

The observed trends in phenol and flavonoid (Table 2) concentrations in BRRI dhan 29 and IR 64 highlight the importance of these secondary metabolites in rice defense mechanisms against *Pyricularia oryzae*. Toan et al. (2017) reported that rice plants infected with *Pyricularia grisea* show increased phenolic and flavonoid concentrations, with greater accumulation in resistant varieties, consistent with the more pronounced increases observed in IR 64. This suggests that IR 64 employs a stronger secondary metabolite-based defense strategy.

The differing antioxidant responses in BRRI dhan 29 and IR 64 reflect their varying capacities to mitigate oxidative stress induced by *Pyricularia oryzae*. The moderate increase in antioxidants in BRRI dhan 29 (Table. 2), indicates limited stress adaptation, whereas IR 64's significantly rises, especially at 120 hours after inoculation, it discloses a robust defense mechanism against *Pyricularia oryzae*. Toan et al. (2017) demonstrated that antioxidant capacity increases significantly in resistant rice varieties under blast stress due to enhanced biosynthesis of protective metabolites like flavonoids and phenolics. Anushree et al. (2016 b) also emphasized that resistant genotypes show elevated activity of defense enzymes and antioxidants, reinforcing their ability to combat fungal infection.

The progressive increase in protein concentration in both BRRI dhan 29 and IR 64 following inoculation with *Pyricularia oryzae* suggests an induced response to pathogen stress. The more pronounced protein accumulation in IR 64 (Table.2) aligns with findings by Yang et al. (2024), where resistant rice cultivars displayed elevated protein synthesis and enzymatic activity during pathogen infection.

The relatively steady sugar levels of BRRI dhan 29 indicate a limited utilization of carbohydrates for defense. The observed increase in sugar levels in IR 64 (Table 2) aligns with findings by Morkunas and Ratajczak. (2014), who highlighted the role of sugar signalling in modulating plant defense responses. Bezrutczyk et al. (2018) also emphasized that sugars, beyond their metabolic role, act as key signalling molecules in plant-microbe interactions, triggering defense responses and reinforcing plant immunity.

Chapter VI

Conclusion and Recommendation

The study effectively determines the responses of different defensive enzymes and non-enzymatic biochemical compounds involved in the physiological resistance of rice cultivars BRRI dhan29 and IR 64 under *Pyricularia oryzae* stress.

Superoxide Dismutase (SOD), Phenylalanine Ammonia-Lyase (PAL) and β-1,3-glucanase activity was increased consistently in both rice cultivars, BRRI dhan29 and IR64, with increase of inoculation time. Though IR 64 exhibited significantly higher SOD activity compared to BRRI dhan29 from 72 to 120 hai, indicating a stronger defensive enzymatic capacity and potentially greater resilience to stress.

There was no significant difference in lipoxygenase (LOX) and chitinase activity levels between the two genotypes. This suggests that both BRRI dhan29 and IR64 exhibit similar LOX and chitinase mediated responses to stress over time.

Initially, H_2O_2 levels of both cultivars were similar but BRRI dhan29 exhibited significantly higher H_2O_2 concentrations at 96 and 120 hours compared to IR64. This suggests that BRRI dhan29 faced more oxidative stress against *P. oryzae* compared to IR 64.

IR 64 exhibited a more pronounced and significant increase of phenol, flavonoid, antioxidant, protein and sugar against *P. oryzae* stress compared to BRRI dhan29 indicating better resilience and adaptive capacity of IR 64 compared to BRRI dhan29.

BRRI dhan29 demonstrated lower enzymatic and non-enzymatic biochemical defense responses compared to IR64 under *Pyricularia oryzae* stress. As BRRI dhan29 is a widely cultivated mega variety, future research should focus on enhancing its stress tolerance mechanisms. This can be achieved through some approaches such as genetic modification, marker-assisted breeding, or the application of biostimulants to strengthen its enzymatic defense responses. Since the current experiment was conducted for only one season, further studies are recommended across multiple

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