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Antioxidant enzyme reprogramming, ROS scavenging, and modulations in secondary metabolites attenuate immunity in rice against *Helminthosporium oryzae* attack

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Abstract. In agricultural ecosystems, plant pathogens cause devastating diseases that jeopardize global food security. This study examined the physiological and biochemical mechanisms of resistance in four rice lines and one variety against *Helminthosporium oryzae*. A completely randomized design experiment revealed significant shoot length reduction due to fungal toxicity, with Kharamana and Sakh showing the least damage (8.3 and 14.76%, respectively), while Binicol suffered the most. Kharamana maintained the highest chlorophyll-a levels indicating post-infection superior tolerance. Antioxidant assays showed increased superoxide dismutase (SOD) activity in Kharamana (56.19%) and Sakh (46.98%), while Binicol exhibited minimal peroxidase (POD) activity. Binicol's ascorbic acid levels dropped by 68.1%, making it highly susceptible. Secondary metabolites, including flavonoids, anthocyanins, and lignin, were significantly altered ($p < 0.05$), influencing resistance. Vulnerability of Binicol was due to lower metabolite levels, whereas Kharamana and Sakh exhibited reprogrammed antioxidant responses, effectively mitigating *Helminthosporium oryzae* effects. This comparative analysis identifies key metabolic pathways for breeding disease-resistant rice, contributing to sustainable agriculture and improved crop management. Identification of these metabolic pathways can contribute to more sustainable and resilient agricultural practices. Additionally, these findings can be extended to rice immunity to other disease for better crop management and advancing crop production.

Keywords: brown leaf spot, enzymes, *Helminthosporium*, immunity, oxidative stress, plants, secondary metabolites

1. INTRODUCTION

Plants have continuously been facing biotic and abiotic pressures since their colonization on the earth (Yang *et al.*, 2022). In agricultural ecosystems, plant pathogens such as viruses, bacteria, fungi, and oomycetes cause devastating diseases that jeopardize global food security as well as plant diversity. The plant-pathogen interactions depend on the ability of plants to detect and respond to pathogen invasion. Both plants and pathogens adopt complex strategies to outmaneuver each other, resulting in a continuous battle between infection and immunity. Successful detection, signaling, and defense execution determines the fate of the plant. Pathogen invasion and resultant incapacitation of plant immunity dampens host plants and reinforces considerable obstacles to plant growth and development (Wang *et al.*, 2022).

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Nature has endowed plants with a sophisticated immune system that includes perception of threat, defense activation, and surveillance for fending off the attacker(s) (Noman *et al.*, 2018c; Chhabra *et al.*, 2019). The plant immune processes are not isolated events. Rather, these defense events are co-regulated by a number of processes. The defense process involves various physiological adjustments, biochemical changes, receptor-medicating signaling, and molecular reprogramming. All of these occur in a highly dynamic, spatially and temporally regulated manner (Tarkowski *et al.*, 2020; Çelik *et al.*, 2020; Chhabra *et al*., 2019; Li *et al.*, 2020). Therefore, no immunological event in plants is random. Each event is a well-coordinated response and finely tuned to the type as well as severity of the attack. In contrast, pathogens also produce secondary metabolites and toxins that interfere with plant growth and disrupt the normal life cycle (Puyam *et al.*, 2017). Pathogen-produced toxins degrade chlorophyll, impair photosynthesis, and stunt plant growth, influencing resistance or susceptibility. Enzymatic antioxidants (SOD, POD, APX), phenol-synthesizing enzyme PAL, and pathogenesis-related proteins play crucial roles in enhancing plant defense against pathogen invasion (Panda, 2007; Chhabra *et al.*, 2023). Together, these components aid in scavenging reactive oxygen species (ROS) and mitigate further cellular damage by triggering the release of defense-related metabolites (Akhter *et al.*, 2024).

Rice (*Oryza sativa*) is a vital cereal crop fulfilling nutritional needs of billions of people across the globe (Khush and Jena, 2009; Das *et al.*, 2023). In Pakistan, *O. sativa* ranks second in cultivation, following wheat (Abbas *et al.*, 2011; Akhter and Haider, 2020). However, the growth and productivity of rice crop are continuously threatened by both biotic and abiotic factors. Of these, biotic stresses impose the most significant challenges to rice growth, development, and production. The primary constraints on rice production are diseases, which undermine both the quantity and quality of the crop. Notable pathogens include bacterial leaf blight (BLB), sheath blight, brown leaf spot (BLS), and false smut. Besides, some insect-pests such as the brown plant hopper and the yellow stem borer also cause destruction of rice crop (Singh *et al.*, 2020). These diseases contribute to an estimated 15-30% yield loss, resulting in an economic burden of approximately $33 billion annually. In extreme cases, they have the potential to cause complete crop failure (Nayak *et al.*, 2021). Brown leaf spot (BLS) disease caused by the fungus *Helminthosporium oryzae* (Sunder *et al.*, 2014) is a prevalent disease in rice crop (Khalil *et al.*, 2014). This disease can cause severe losses, with crop damage reaching as high as 90% in severe outbreaks (Barnwal *et al.*, 2013; Muimba-Kankolongo, 2018). A historical example of its catastrophic impact was the Bengal famine of 1942-43 triggered by *H. oryzae*, which resulted in the deaths of around 2 million people (Lenné, 2000; Haydock *et al.*, 2021). Initially, BLS appears as small brown lesions, which progress into oval, cylindrical, or round shapes, resembling sesame seeds. As these lesions coalesce, they form large patches ranging from 0.5 and 2.0 mm wide. Primarily, the *H. oryzae* infection spreads across leaves causing the formation of reddish-brown lesions. As the disease progresses, it hampers plant growth, leading to reduced grain yield and seed discoloration (Lore *et al.*, 2015). The rising demand for rice, driven by population growth and urbanization, underscores the need for sustainable production amid economic vulnerabilities in Asia and Africa. Meeting future demands requires continuous and advanced research to close yield gaps and minimize post-harvest losses, as well as abiotic stresses and pathogen-induced yield reductions.

Rice disease management involves development of resistant varieties, application of cultural practices, and the use of chemical or biological controls. While chemical control is highly effective in mitigating the severity of BLS disease, these methods have significant drawbacks. Moreover, the overuse and unregulated application of agrochemicals can jeopardize both environmental integrity and human health. Among the various strategies, the use of resistant varieties is the most widely recognized approach for managing BLS in a sustainable and long-lasting way (Barnwal *et al*., 2013; Van Bockhaven, 2014). Typically, the breeding of new varieties aims to increase both productivity and disease resistance. However, this challenge becomes particularly formidable when dealing with pathogens that reproduce sexually. Additionally, the increasing genetic diversity of pathogens and the likelihood of resistance against chemicals used for pathogen control create problems of gigantic magnitude. For effective resistance to BLS, it is critical to harness the innate immune traits of host plants that contribute to their defense against pathogens. Metabolic changes in infected plants result in the production of compounds, *e.g*., phenols and phytoalexins, that prepare plants to fight and resist pathogens in a natural way (Torres *et al.*, 2006; Kaur *et al.*, 2022). A thorough understanding of these physiological and biochemical changes could pave the way for the development of new rice varieties with improved resistance against *H. oryzae* invasion or colonization. Hence, we hypothesized that changes in antioxidant levels and secondary metabolite production differentially contribute to the immunity of new rice lines against *H. oryzae*. Therefore, the main objective of this study was to explore the physiological and biochemical mechanisms underlying resistance in newly developing rice lines against *H. oryzae* and to validate the resistance mechanisms. Understanding the physiological and biochemical traits associated with resistance to *H. oryzae* could facilitate the identification of genes responsible for conferring resistance to this pathogen in rice.

2. MATERIALS AND METHODS

A field experiment was conducted to evaluate the susceptibility and resistance of rice lines to *H. oryzae*. The experiment evaluated the impact of pathogen infection on the growth and diverse physio-biochemical attributes of four newly developed rice lines, alongside one established variety of rice (*O. sativa*) selected on the findings of our previous study (Akbar *et al*., 2023) (Table 1). These rice lines were obtained from the International Rice Research Institute, Manila, Philippines. Rice nursery was established and 21-day-old plants were transplanted in the field. Regular irrigation was maintained in the field along with supply of NPK. After 20 days of transplant, rice plants were subjected to pathogen attack. Weeding was performed in regular intervals.

Table 1. List of rice lines and their associated codes used for the experiment (Akbar *et al*., 2023)

Code	Name
V1	Sakh
V2	Campena
V3	Kharamana
V4	KSK-282
V5	Binicol

The experiment was designed in a completely randomized design with five replications in natural atmospheric conditions. During experimentation, the average weather conditions were as follows:
Average temperature = 35±3°C, Relative humidity = 51±4, Average day length = 14h.

2.1. Isolation, purification, and multiplication of *H. oryzae*

The extent of yield loss in rice is influenced by various factors, including rice varieties, environmental conditions, and infection levels. *Helminthosporium oryzae* was isolated from rice plants showing symptoms of brown leaf spot disease, *i.e.* cylindrical to oval brown spots that later became black, in the Faisalabad region of Pakistan in 2021 and 2022. The fungus was collected, confirmed, and later propagated as per protocol already described by Parada *et al*. (2015) and Akbar *et al*. (2023). The purified *H. oryzae* culture was regrown on potato dextrose agar (PDA) media. The distinguished characteristics of colonies included gray to dark greenish gray mycelium exhibiting fluffy and cottony growth. The hyphae were septate and gray-greenish in color. Brown conidia were widest in the middle and tapering to rounded ends (Supplementary Fig. 1). The optical density (OD600) of the freshly prepared inoculum having spores was adjusted to 0.6 OD using a UV-visible spectrophotometer (Model Hitachi-U 2001, Tokyo Japan) (Shamshad *et al*., 2024; Ashfaq *et al*., 2021, Parada *et al*., 2015). The standardized inoculum was then sprayed onto individual plants in the experimental field.

2.2. Growth attributes

To assess the impact of *H. oryzae* on rice growth, various growth parameters, including shoot and root length, root and shoot fresh weight, and root and shoot dry weight, were evaluated in 48-day-old rice plants.

2.3. Chlorophyll contents

The chlorophyll-a and b contents were determined using an established protocol (Arnon, 1949) (Supplementary info 1).

2.4. Quantification of anthocyanin

The anthocyanin contents were quantified using the methodology outlined by Mahajan *et al.* (2011) (Supplementary info 2).

2.5. Lignin determination

Lignin estimation was performed with a method described by Klason (1923), *i.e.* a gravimetric method that measures insoluble material remaining after hydrolysis with 72% sulfuric acid (H_2SO_4). It is often combined with spectrophotometric determination of dissolved lignin.

2.6. Estimation of flavonoids and phenolics

The total flavonoid contents in the shoot system were determined by following the method proposed by Zhishen *et al.* (1999) while total phenolics in leaves were determined by following the method described by Julkunen-Tiitto (1985) (Supplementary info 3, 4).

2.7. Total amino acid quantification

Total free amino acids were estimated as in Hamilton *et al.* (1943) (Supplementary info 5).

2.8. Hydrogen peroxide (H_2O_2) and lipid peroxidation measurement

The H_2O_2 content in plant samples was measured as described by Velikova *et al.* (2000) and Alexieva *et al*. (2001). Using a standard curve made with known H_2O_2 values, the quantity of hydrogen peroxide was determined.

2.9. Malondialdehyde estimation

The method developed by Cakmak and Horst (1991) was employed to determine the levels of malondialdehyde (MDA) in plant tissues. Fresh leaves (0.5 g) were homogenized in a 10 mL solution of 0.1% (w/v) trichloroacetic acid (TCA), followed by centrifugation at 12 000 × g for 10 min. Subsequently, to the supernatant, 4.5 mL of thiobarbituric acid (0.5%) was added to 1 mL of the extract. Then, the

reaction mixture was heated at 95°C in a water bath for 30 min. After cooling, the sample was again centrifuged, and the absorbance readings were recorded at wavelengths of 532 and 600 nm using a spectrophotometer (Model Hitachi-U 2001, Tokyo Japan).

2.10. Antioxidant enzyme activities

Antioxidant enzymes were extracted from fresh leaves (0.5 g), which were finely ground in 10 mL of ice-chilled 5 mM potassium phosphate buffer (pH 7.4). The homogenate was centrifuged at 12 000 × g at 4ºC for 20 min using a centrifuge (Sigma model 3K30, Germany). The supernatant was re-centrifuged for 10 min at 15000 × g, and the extract was then stored at -20°C for further analysis of antioxidant enzymes.

2.11. Determination of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activity

Superoxide dismutase (SOD) activity was determined using a method proposed by Giannopolitis and Ries (1977), based on the photochemical reduction of nitroblue tetrazolium (NBT). Absorbance was measured at 560 nm with the help of a UV visible spectrophotometer (Hitachi U-2100, Tokyo, Japan). Additionally, on a protein basis, the activity of all antioxidant enzymes was determined. The activity of POD was determined by following the method used by Chance and Maehly (1955) with some modifications. For peroxidase (POD) activity, a mixture consisting of 750 µL of phosphate buffer (pH 5.0 and 50 mM), 100 µL of 20 mM guaiacol, 100 µL of H_2O_2, and 100 µL of enzyme extract was used. The change in the absorbance value for the reaction mixture was read at 470 nm, and reading was taken after every 120 s with the help of a spectrophotometer (Hitachi-U2001, Tokyo, Japan). For estimation of catalase (CAT) activity, 3 mL of a reaction solution was prepared (1.9 mL of 50 mM phosphate buffer (pH 7.0), 1 mL of 5.9 mM H_2O_2, and 0.1 mL of enzyme extract). After adding the aliquot enzyme, the extract reaction was initiated. Enzymatic activity was monitored at 240 nm using the spectrophotometer after every 20 min.

2.12. Polyphenol oxidase (PPO) activity assay

Polyphenol oxidase (PPO) activity was measured by following the protocol proposed by Esterbauer *et al.* (1977). 100 mg of fresh leaves of pathogen-infected and control rice plants were ground in liquid nitrogen to a fine powder. 1 mL of extraction buffer containing 0.1 M Tris-HCl (pH 7.0), 0.1 M KCl, 1% (v/v) Triton X-100, 1 mM EDTA, and 5% (w/v) PVPP (polyvinylpolypyrrolidone) (Thipyapong *et al.*, 1995) was used in the extraction. After centrifuging the extract for 15 min at 4°C and 12,000 × g, the supernatant was transferred to a fresh tube. Bradford's technique (1976) was used to quantify the protein content in the extract. Upon noticing a decline in the absorbance of 2-nitro-5-thiobenzoic acid at 412 nm following a coupled reaction with quinones produced by the enzyme oxidation of 4-methylcatechol, PPO activity was measured spectrophotometrically (Esterbauer *et al*., 1977).

2.13. Phenyl alanine lyase (PAL) and tyrosine alanine lyase (TAL) activity

Assays for phenyl alanine lyase (PAL) activity, protein extraction were conducted in accordance with the protocols provided by Cheng and Breen (1991) with slight modifications. After grinding, frozen plant material (0.2 g) was treated with 1 mL of acetone and incubated for 15 minutes at -20°C. Then, the mixture was centrifuged for 15 min at 4°C and 16 000× g. Low-speed spinning at 4°C with 100 mM sodium borate (pH 8.8) and 2 mM EDTA solution produced pellets. A second centrifugation was performed after 60 min and supernatant was obtained. The plant extract and buffer solution without substrate was used as a blank. Standards were made using L-phenylalanine and L-tyrosine as substrates and trans-cinnamic acid (TCA) and p-coumaric acid (PCA) as products. The synthesis of TCA was evaluated by measuring absorbance at 290 nm (spectrophotometer UV-3600, Shimadzu, Kyoto, Japan) for PAL activity. The supernatant made for PAL activity was used for TAL activity measurement. The supernatant was run over a Sephadex G-25 column that had been previously equilibrated with a 0.01 M borate buffer solution (pH 8.8) using identical procedures as those used for determining PAL activity mentioned above. The real p-coumaric acid (Sigma Chemical Co.) standard curve was employed.

2.14. Quantification of ascorbate peroxidase and ascorbic acid

Ascorbate peroxidase (APX) was measured using the methodology outlined by Wise and Naylor (1987). 0.5 g of frozen leaves were pulverized with the help of a pestle and mortar, and 5 mL of 6% (v/v) $HClO_4$ was added and the mixture was centrifuged at 10 000 × g for 10 min. A 100 mL aliquot of leaf extract was immediately mixed with 900 mL of 200 mM succinate buffer (pH 12.7, adjusted with NaOH). The leaf extract was centrifuged at 10,000 × g for 5 min after being adjusted to pH 6.0 with 1.25 M K_2CO_3. This allowed the estimation of total ascorbate peroxidase. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH buffer (pH 7.5) was incubated for 10 min at 25°C with 10 mM dithiothreitol added to the supernatant. 900 mL of 200 mM succinate buffer (pH 6.0) was filled with a 100 mL aliquot of the solution. The resulting solution underwent the absorbance measurement at 290 nm using a spectrophotometer.

Ascorbic acid was quantified according to the method described by Barata-Soares *et al.* (2004). The samples were homogenized using a 0.1% solution of metaphosphoric acid in the prescribed amounts after being ground in liquid nitrogen. Subsequently, the homogenate underwent 10 min

centrifugation at 12,000 × g. Then, by using a 0.45 mm millipore membrane, the supernatant was filtered and diluted with dithiothreitol for total ascorbic acid determination. 0.2 mol L^{-1} acetate buffer pH 4.5 was used as the mobile phase in the HPLC analysis of the extracts using a Bondapak C18 column at a flow rate of 1.5 mL min^{-1}. At 254 nm, chemicals that were eluting from the column were found. A standard curve spanning from 10 to 100 mmol was obtained per liter of AsA.

2.15. Estimation of changes in the rice ionome

The ionome of rice was assessed by digesting 100 mg of oven-dried, shredded leaf material in a 10 mL Pyrex glass vial containing 1 mL of the digesting mixture (14.0 g $LiSO_4$, 0.42 g Se, 350 mL H_2O_2 (35% AR grade), and 0.5 mL perchloric acid ($HClO_4$)). The vials were incubated on a heated plate within a fume hood, with the temperature maintained at 350°C. Digestion was considered complete when the mixture became colorless. The resulting extract was filtered, and double-distilled water was added to maintain its final volume of 50 mL. The concentrations of magnesium (Mg), phosphorus (P), sodium (Na^+), potassium (K^+), and calcium (Ca^{2+}) were measured in accordance with Allen *et al*. (1985).

2.15.1. Determination of calcium, potassium, magnesium, and sodium

The concentrations of mineral nutrients (Ca^{2+}, K^+, Mg^{2+}, and Na^+) were determined using a double-channel flame photometer (Model Jenway, PFP-7, UK). Highly pure standards (5 to 60 mg L^{-1}) were used to generate standard curves for each nutrient. The concentrations of the nutrients in the samples were calculated based on these standard curves, following the protocol outlined by Wolf (1982).

2.15.2. Determination of phosphorus (P)

Phosphorus (P) was determined using Barton's reagent, prepared according to Jackson (1958). To create the reagent, 12.5 g of ammonium molybdate was dissolved in 200 mL of distilled water. In a separate beaker, 0.625 g of ammonium metavanadate was dissolved in 300 mL of boiling water and allowed to cool, followed by the addition of 250 mL of concentrated HNO_3. The two solutions were mixed thoroughly, cooled, and the final volume was adjusted to 500 mL. For the analysis, 1 mL of the filtrate was mixed with 1 mL of Barton's reagent, followed by the addition of 25 mL of distilled water. The mixture was allowed to stand for half an hour before being analyzed using a spectrophotometer at 470 nm. The calculation of P values was done by using a standard curve.

2.16. Statistical analysis

The data collected in this study were statistically analyzed using the COSTAT (6.303) statistical software (Cohort Software, Monterey, CA, USA). The results are presented as mean values ± standard error (SE) and depicted in bar plots to provide a clear visual representation of the data. To assess the impact of the *H. oryzae* application and the control treatment on various studied attributes across five rice lines and a variety (V1 to V5), two-way analysis of variance (ANOVA) was performed. Following the ANOVA, Tukey's Honestly Significant Difference (HSD) post-hoc test was applied to conduct multiple comparisons and identify specific differences between the treatments. Statistically significant differences among the rice lines were indicated by different letters placed above the bars in the plots ($p < 0.05$), highlighting where significant variations occurred post and pre- *H. oryzae* application. All graphs were generated using Origin Pro version 2021 software (Origin Lab Corporation, 2021).

3. RESULTS

3.1. Growth parameters

The analysis of variance (ANOVA) for growth attributes revealed significant ($p \leq 0.05$) variation across all the rice lines. Among them, V3 exhibited the highest shoot fresh weight in both the control and pathogen attack conditions, outperforming the other rice plants (Fig. 1). Under *H. oryzae* attack, the smallest reduction in shoot fresh weight was observed in V3 (23.2%) and V4 (23.1%) compared to the other plants in the study. In contrast, V1 experienced a comparatively high loss in fresh weight due to *H. oryzae* attack relative to the control. For shoot dry weight, V5 exhibited the lowest values in both the control and pathogen attack conditions. Among the pathogen-stressed plants, V4 showed a 71.4% decrease in dry weight, while V5 experienced a 70.2% reduction compared to the control. Kharamana (V3) recorded the highest root fresh weight in both the control and *H. oryzae*-infection conditions. However, post-infection, all the rice types showed a reduction in root fresh weight, with reductions of 25.3, 19.5, 7.8, 18.6, and 42.5%, respectively. Binicol (V5) experienced the greatest losses in both root fresh and dry weights, indicating that it was the most adversely affected rice line, while V1, V2, and V4 showed similar responses in both stressed and controlled environments.

The statistical analysis of the data revealed significant variations ($p \leq 0.05$) in both shoot and root lengths among the different rice lines studied. The maximum shoot length was observed in V3, both in the presence and absence of *H. oryzae* attack, indicating its superior growth performance. In contrast, V5 exhibited the greatest reduction in shoot length in both the control and pathogen-infection conditions, highlighting its high susceptibility. The *Helminthosporium oryzae* attack had a more pronounced effect on root length compared to shoot length. The highest root length in both the controlled and infection conditions was consistently shown by V3, reflecting its resilience. Nearly identical root lengths across the control and infection

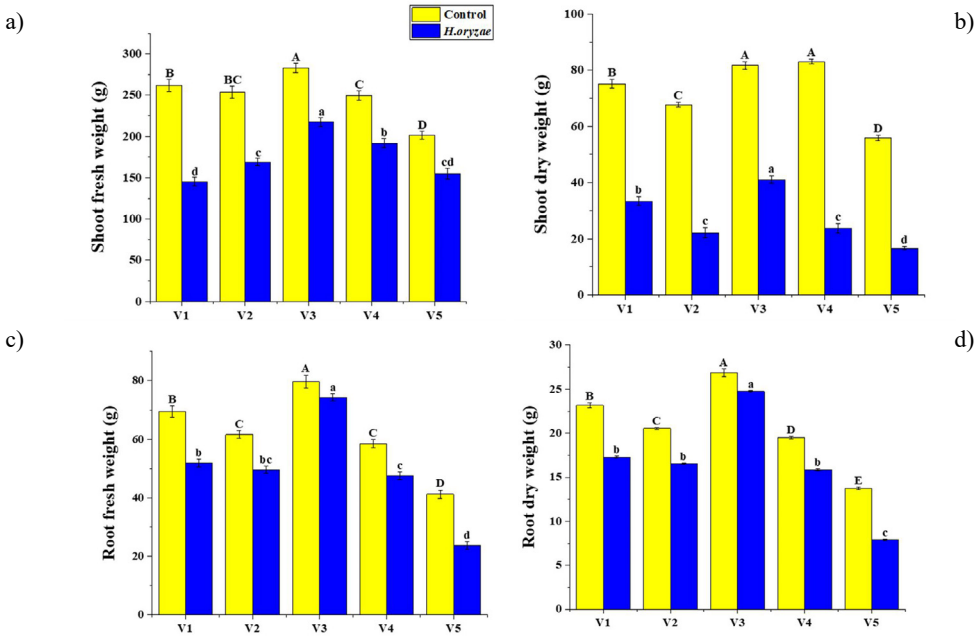

Fig. 1. Comparative morphological attributes of newly established rice lines and variety under control and *Helminthosporium oryzae* infection: a) shoot fresh weight, b) shoot dry weight, c) root fresh weight, d) root dry weight. Letters above the bars depict significant differences between the rice lines. Bars having different letters denote statistically significant behavior. Capital letters represent differences among means in infected rice, while small letters show differences among means in non-infected plants ($n = 5$, $p \leq 0.05$). (V1 = Sakh, V2 = Campena, V3 = Kharamana, V4 = KSK-282, V5 = Binicol).

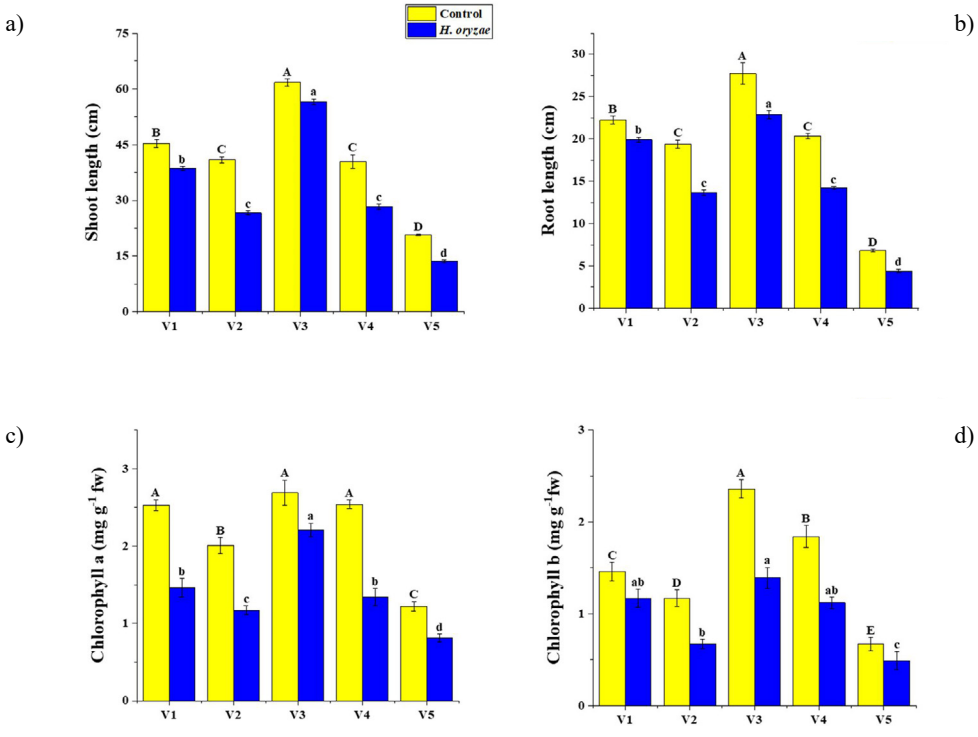

Fig. 2. Comparative morphological and biochemical attributes of newly established rice lines and variety under control and *Helminthosporium oryzae* infection: a) shoot length, b) root length, c) chlorophyll a, d) chlorophyll b. Letters above the bars depict significant differences between the rice lines. Bars having different letters denote statistically significant behavior. Capital letters represent differences among the means in infected rice, while small letters show differences among means in non-infected plants ($n = 5$, $p \leq 0.05$). (V1 = Sakh, V2 = Campena, V3 = Kharamana, V4 = KSK-282, V5 = Binicol).

scenarios were displayed by V2 and V4, suggesting similar responses to pathogen stress. V5 demonstrated the lowest root length among all the lines, with a 35.48% reduction in the control conditions, which was further exacerbated by the *H. oryzae* infection (Fig. 2).

3.2. Chlorophyll contents

A significant ($p \leq 0.05$) diminution in the chlorophyll a content in the studied rice lines and the variety after the pathogen attack was documented by ANOVA. V5 was the most severely affected, showing a 47.2% reduction in chlorophyll a levels. In the control conditions, lines V1, V3, and V4 exhibited nearly identical chl. a values with non-significant differences. However, following the infection, highly significant variations emerged among these lines (Fig. 2).

The exposure of the rice plants to *H. oryzae* revealed significantly ($p \leq 0.05$) different observations for chlorophyll b among all the studied rice plants. V3 and V4 showed the highest chlorophyll b content in both the normal and infection conditions indicating relatively better resilience to disease attack. In contrast, rice line V5 consistently recorded the lowest chlorophyll b levels in both conditions. Overall, the pathogen attack caused maximum destruction in chlorophyll b in V2 and V3 (-42.7 and 41.1%, respectively) (Fig. 2).

3.3. Anthocyanins, lignin, flavonoids, and phenolics

The statistical analysis revealed significant ($p \leq 0.05$) variations in the anthocyanin, lignin, and flavonoid contents among the studied rice lines in the control and after the *H. oryzae* infection. In the control conditions, V3 exhibited the highest anthocyanin content, followed by V4. The pathogen attack caused a decline in anthocyanin levels across all the lines, with V4 experiencing the largest reduction (-26.08%) and V2 the smallest (-3.3%). These changes suggest a pathogen-induced metabolic shift influencing resistance mechanisms. The lignin content was also significantly altered ($p \leq 0.05$) by the infection, with V3 showing the highest levels post-attack, followed by V1 and V2, whereas V5 experienced a substantial decrease (57.6%), potentially compromising its defense response. Similarly, flavonoids decreased significantly ($p \leq 0.05$) upon the infection across all the lines. Notably, V3 showed a non-significant difference between the control and infection conditions, indicating higher resilience, whereas V5 demonstrated the highest susceptibility, with a 73% reduction in the flavonoid content. These findings present V3 as the most tolerant and V5 as the most susceptible line against *H. oryzae* (Fig. 3).

Significant variations in the phenolic content were observed among the rice lines, with V2 and V5 showing highly notable differences compared to the other lines in the control conditions. Upon the pathogen attack, a decline in phenolics was evident across all the lines, with the most pronounced reductions recorded in V3 (-64.2%) and V4 (-37.7%) (Fig. 3).

3.4. Hydrogen peroxide and lipid peroxidation

Significant ($p \leq 0.05$) differences in the hydrogen peroxide (H_2O_2) levels were observed between the control and infected plants. In both conditions, V5 exhibited the highest H_2O_2 content, followed by V1. In contrast, V3 and V4 displayed no notable differences in the H_2O_2 levels. Upon the bacterial inoculation, a 12.09% reduction in the H_2O_2 content was recorded in V1, while V3 and V5 experienced a substantial increase of 61.7 and 66.6%, respectively, indicating a heightened oxidative response in these lines (Fig. 4).

The analysis of variance also revealed a significant ($p \leq 0.05$) rise in the malondialdehyde (MDA) content under the pathogen attack. Among the infected plants, V5 and V1 recorded the highest MDA levels reflecting severe lipid peroxidation. In contrast, V2, V3, and V4 exhibited similar trends with moderate increases. Specifically, the MDA content increased by 53.3% in V1 and 46.1% in V3 following the *H. oryzae* infection. In the non-infected control conditions, the highest MDA levels were again observed in V5 followed by V2. The lowest MDA content was recorded in V3, highlighting its better resilience in normal conditions (Fig. 4).

3.5. Enzymatic and non-enzymatic antioxidants

Superoxide dismutase (SOD) activity was measured in rice plants following the pathogen infection, revealing an increase compared to the non-infected plants. V5 exhibited the lowest superoxide dismutase (SOD) activity, while V3 consistently maintained the highest levels. Reflecting their resilience to pathogen attack, V4 and V3 showed significant increases in SOD activity by 56.19% and 46.98%, respectively. Even under the *H. oryzae* infection, V3 retained the highest SOD activity, whereas V5 demonstrated the greatest susceptibility, indicated by its persistently low SOD levels (Fig. 4). The analysis of variance revealed a significant ($p \leq 0.05$) increase in peroxidase (POD) activity across all the rice lines in response to the *H. oryzae* infection. Among the non-inoculated plants, V4 recorded the lowest POD activity followed by V2. Notably, V5 exhibited a remarkable 96.12% surge in POD activity after the pathogen attack, whereas the other lines showed no significant changes in their activity levels (Fig. 4).

In the control conditions, V3 demonstrated significantly higher ascorbic acid (AsA) levels than all the other lines. The statistical analysis showed that V1 and V2 had comparable AsA levels ($p \leq 0.05$) in the absence of infection but displayed variable responses following the pathogen exposure. The remaining lines exhibited minimal variation. Post-infection, V5 was the most adversely affected, with a striking 68.1% reduction in AsA levels compared to

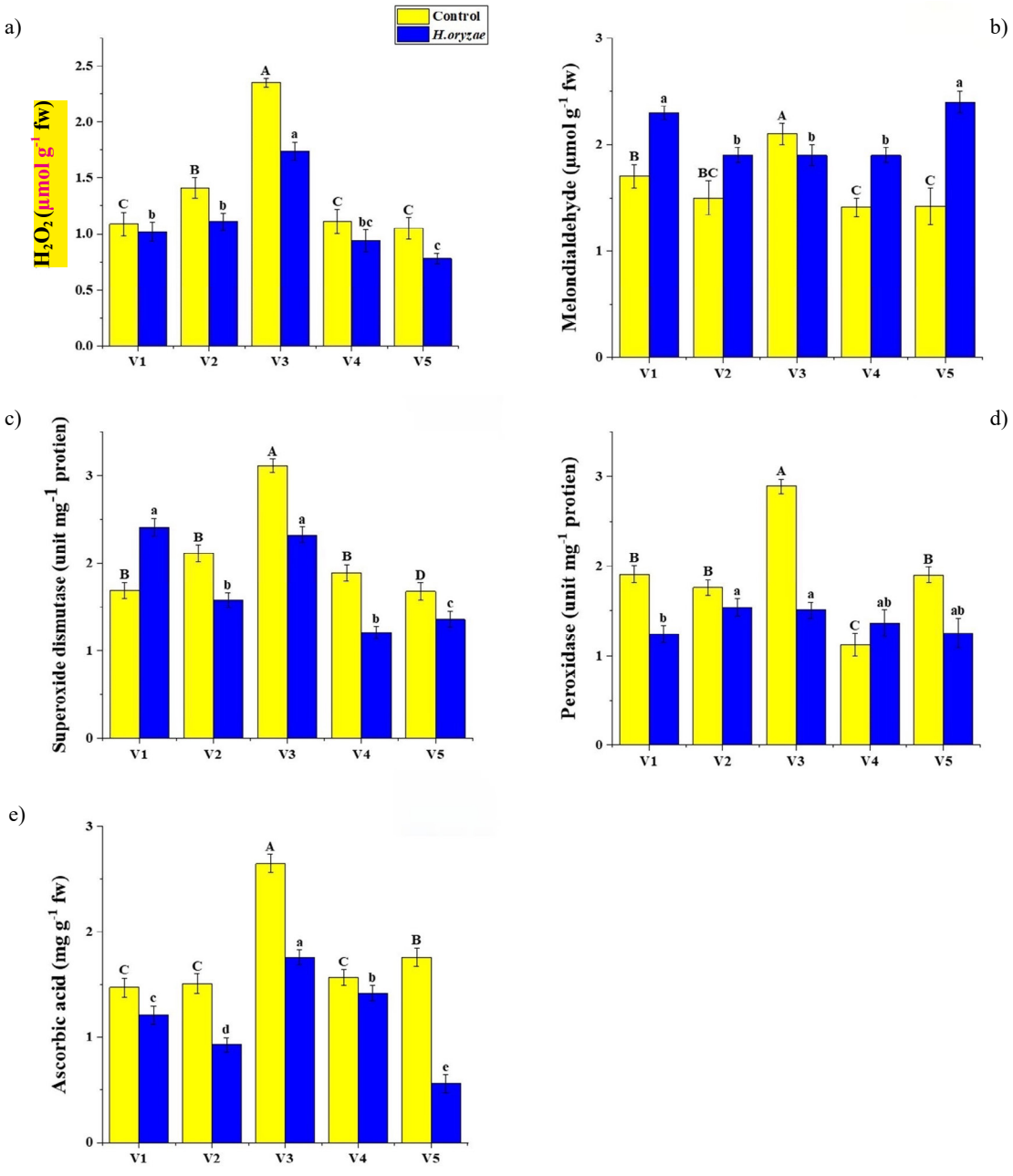

Fig. 3. Estimation of: a) H_2O_2, b) malondialdehyde, c) superoxide dismutase, d) peroxidase, e) ascorbic acid in *Helminthosporium oryzae*-inoculated and non-inoculated plants. Letters above the bars depict significant differences between the rice lines. Bars having different letters denote statistically significant behavior. Capital letters represent differences among means in infected rice, while small letters show differences among means in non-infected plants ($n=5$, $p \leq 0.05$). (V1 = Sakh, V2 = Campena, V3 = Kharamana, V4 = KSK-282, V5 = Binicol).

its control condition. In contrast, V4 showed only a minor decrease of 9.5%, underscoring its relative resilience to *H. oryzae*-induced stress.

The highest ascorbate peroxidase (APX) activity was observed in V3, while V5 displayed the lowest levels among control plants. The pathogen attack had a negative impact on APX activity across the rice lines, with V5 exhibiting the most pronounced and statistically significant decrease compared to the other lines. V1 and V3 demonstrated increases in post-infection APX activity by 13.6 and 14.9%. In contrast, V2, V4, and V5 experienced reductions in APX activity, highlighting their differing responses to pathogen stress (Fig. 5). Catalase activity showed the most substantial increase in V3, with a significant 33.32% rise following the infection. The other lines exhibited relatively stable catalase activity, with no notable post-infection changes. Among the control plants, a declining trend in

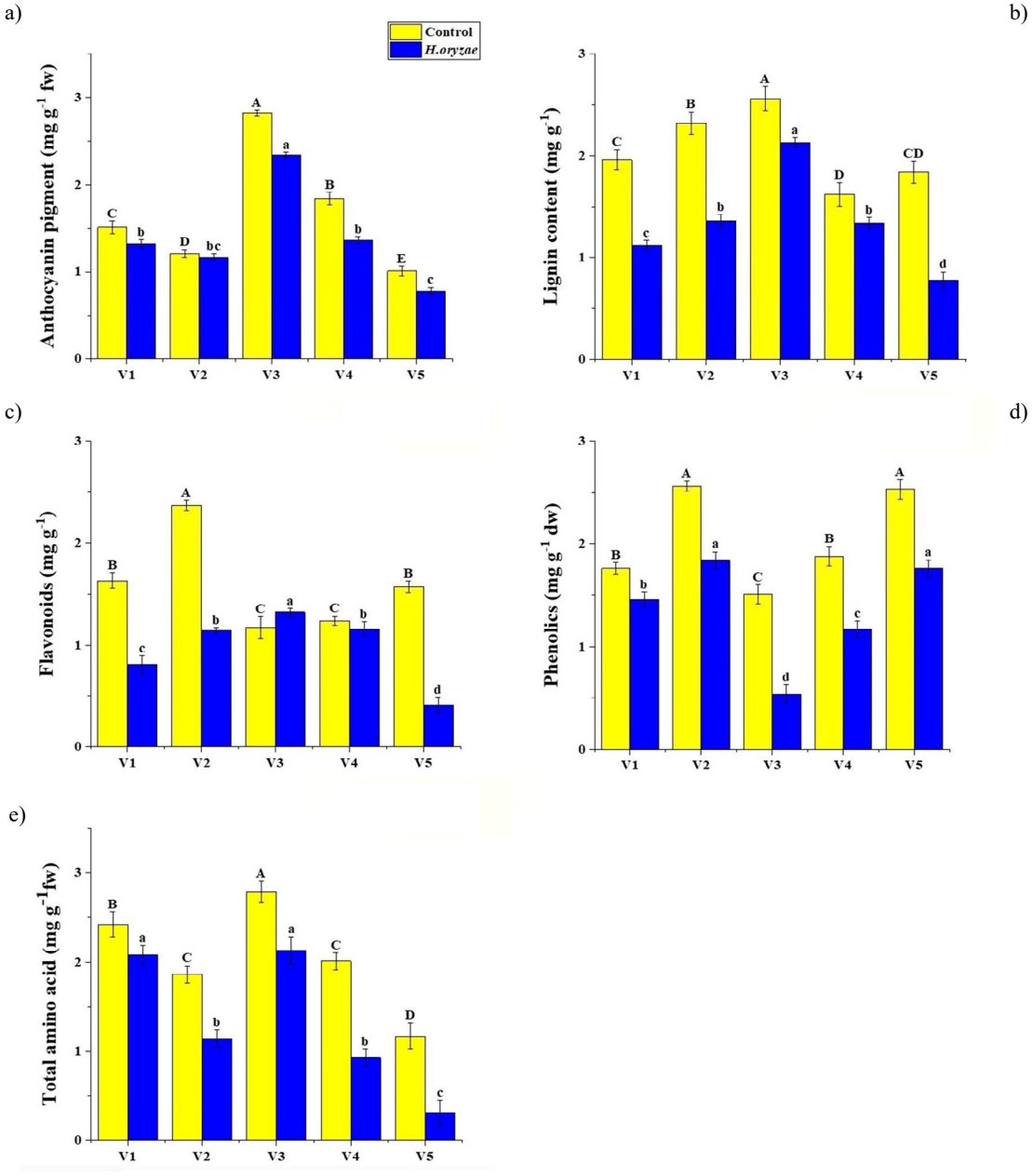

Fig. 4. Changes in: a) anthocyanin, b) lignin content, c) flavonoids, d) phenolics, e) total amino acid contents in rice plants infected with Helminthosporium oryzae compared to control plant leaves. Significant variations across the rice lines are represented by different letters above the bars. Bars having different letters denote statistically significant behavior. Capital letters represent differences among means in infected rice, while small letters show differences among means in non-infected plants ($n = 5$, $p \leq 0.05$). (V1 = Sakh, V2 = Campena, V3 = Kharamana, V4 = KSK-282, V5 = Binicol).

catalase activity was observed from V5 to V2. Overall, the pathogen attack had the most pronounced effect on V3, underscoring its distinct metabolic response to stress.

All the rice lines exhibited gradual increases in PPO activity compared to that in the non-inoculated control plants. Differential and significantly high PPO activity levels were evident in the infected plants compared to the control plants. Consistently low levels of endogenous PPO were present in rice plants grown in the absence of the pathogen. The PPO activity reached its maximum in V1, V3, and V4 and declined thereafter in the control plants (Fig. 5). The highest PPO activity in the *H. oryzae*-infected rice plants was recorded in V4 followed by V1, V2, and V3 (79.85, 76.31, 75.03, and 74.23%). The lowest PPO activity was noted in V5, but it was also (69.28%) higher than its counterpart grown in the control.

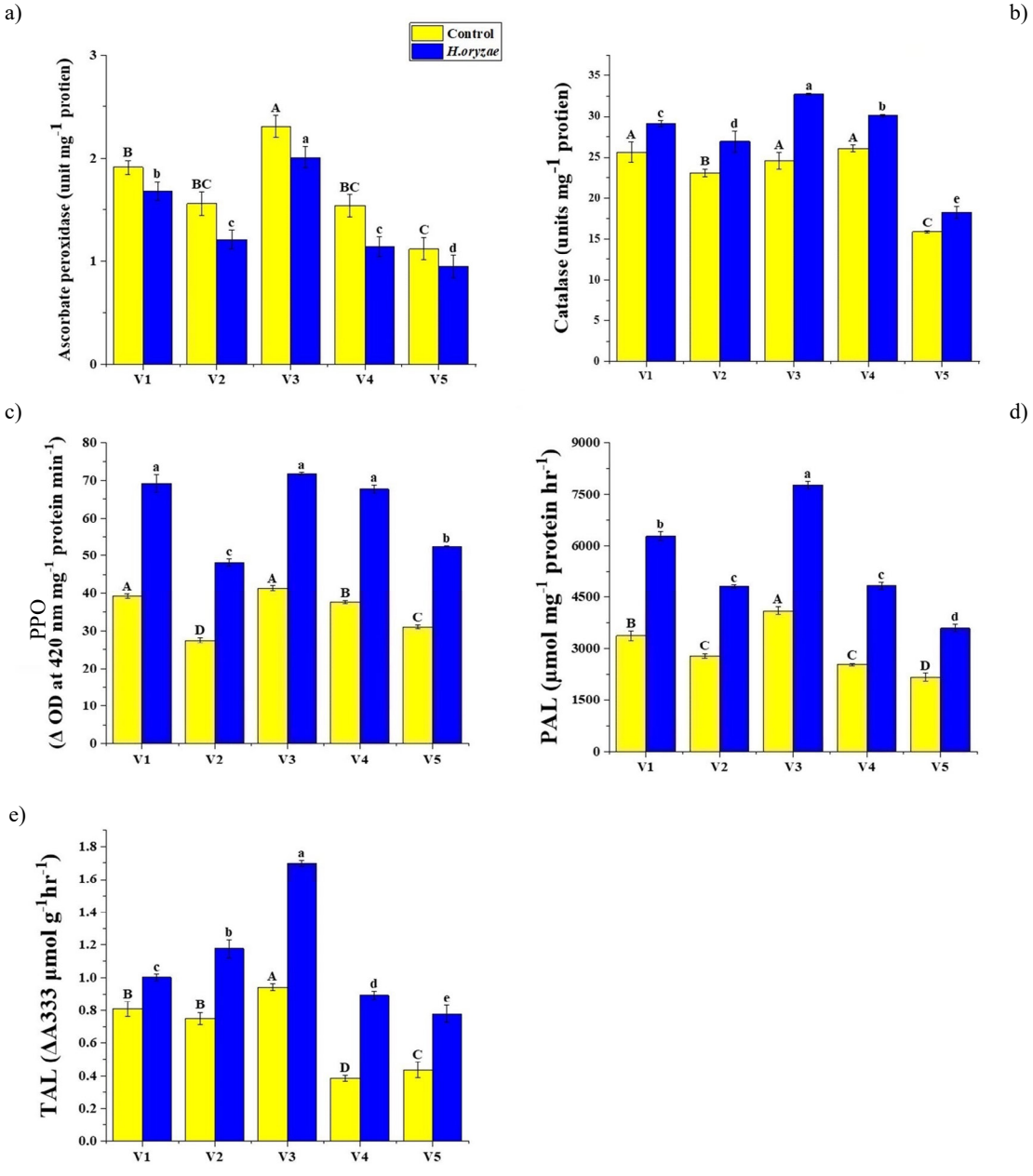

Fig. 5. Estimation of: a) ascorbate peroxidase, b) catalase, c) PPO, d) PAL, e) TAL in *Helminthosporium oryzae*-inoculated and non-inoculated rice plants Letters above the bars depict significant differences between the rice lines. Bars having different letters denote statistically significant behavior. Capital letters represent differences among means in infected rice, while small letters show differences among means in non-infected plants ($n = 5$, $p \leq 0.05$). (V1 = Sakh, V2 = Campena, V3 = Kharamana, V4 = KSK-282, V5 = Binicol).

Upon the pathogen inoculation, phenylalanine ammonia-lyase (PAL) activity increased across all the rice lines. Compared to the non-infected plants, the pathogen-infected rice varieties exhibited substantial and statistically significant ($p \leq 0.05$) changes in PAL activity. Among the studied rice lines, the highest PAL activity was recorded in V3, reaching a peak of 7 770.66 µmol mg^{-1} h^{-1}, while the non-infected V3 plants exhibited a considerably lower PAL activity of 4 110.33 µmol mg^{-1} protein h^{-1}. Under the pathogen attack, the PAL activity in V4 was followed closely by V3 and V1 exhibiting the highest relative increase under the *H. oryzae* infection rising by 90.27, 89.05, and 85.57%, respectively (Fig. 5). Among all the rice lines, the highest TAL activity was determined in V3, while V1 and V2 exhibited comparable levels in the control plants. The infection by *H. oryzae* led to a marked elevation in TAL activity with significant variation across the rice lines. Post-pathogen attack, the highest TAL activity was recorded in V4 followed by V3 with respective increases of 131.6 and 80.1% compared to their controls. A consistent upward

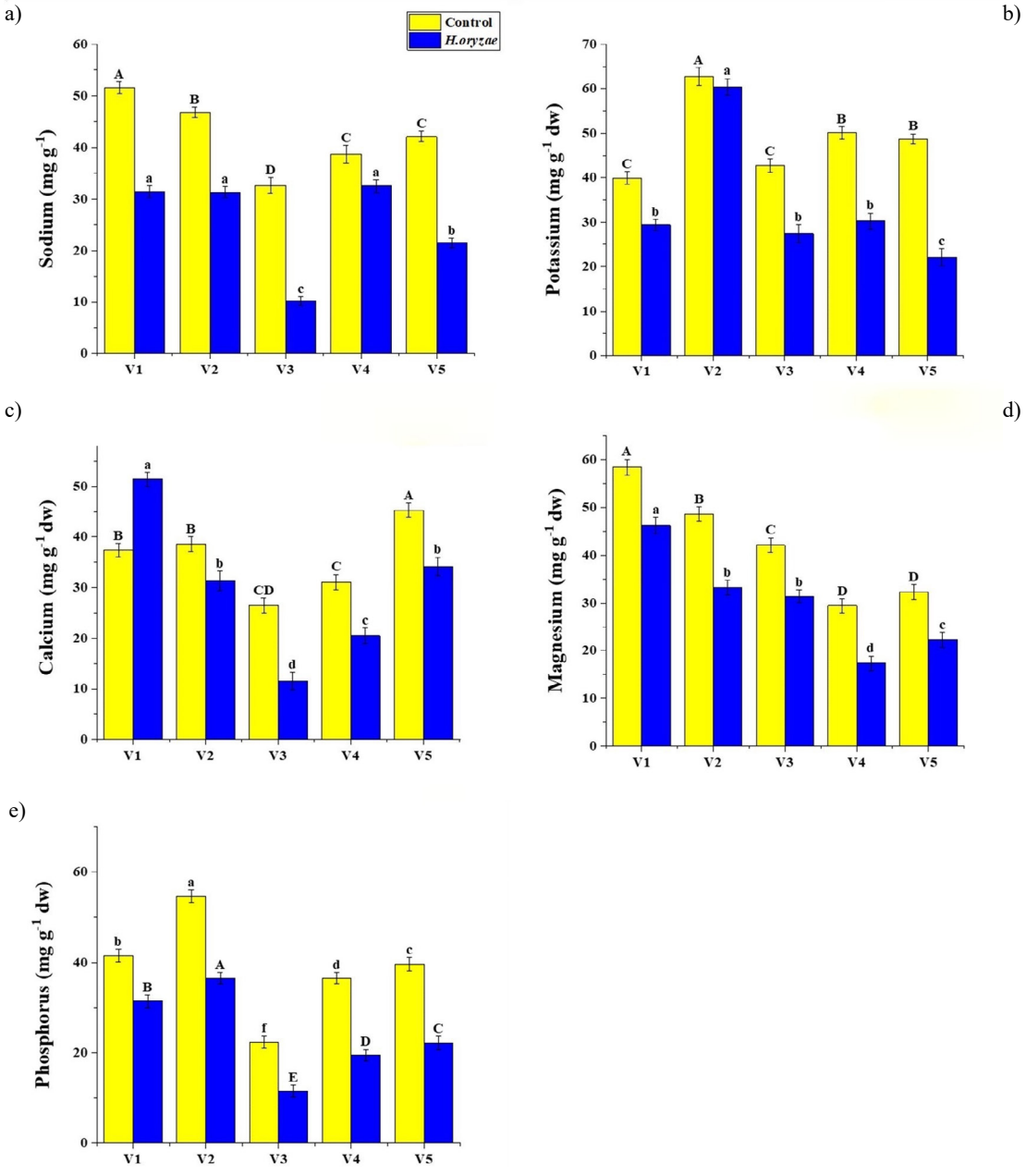

Fig. 6. Estimation of different ions in rice plants: a) sodium, b) potassium, c) calcium, d) magnesium in *Helminthosporium oryzae*-inoculated and non-inoculated plants Letters above the bars depict significant differences between the rice lines. Bars having different letters denote statistically significant behavior. Capital letters represent differences among means in infected rice, while small letters show differences among means in non-infected plants ($n = 5$, $p \leq 0.05$). (V1= Sakh, V2 = Campena, V3 = Kharamana, V4 = KSK-282, V5 = Binicol).

trend in TAL activity was observed across all the rice lines relative to the control. Notably, V4 and V5 having relatively low TAL activity in the control plants showed significant increases of 131.6 and 78.8% following the pathogen infection (Fig. 5).

3.6. Rice ionome

In the control condition, the highest Na level was noted in V1 and V2, while the other lines showed a declining trend. Following the *H. oryzae* infection, the Na levels decreased across all the rice lines, with the most significant reduction recorded in V3 (-68.7%). Other notable reductions were observed in V5 (-48.9%) and V1 (-39.1%) (Fig. 6).

In our findings, potassium (K) levels in V2 remained unaffected by the pathogen infection, while V4 and V5 showed similar responses. However, V5 exhibited the highest reduction in K levels (-54.6%) followed by a decline in V4 and V3. Post-infection, all the rice lines experienced reductions in K levels: V1 (-26.3%), V2 (-3.8%), V3 (-35.8%), V4 (-39.7%), and V5 (-54.6%) (Fig. 6).

In the control conditions, the highest calcium (Ca) level was recorded in V5, V2, and V1, with V3 showing the lowest values. Upon the pathogen attacks, all the rice lines except V1 experienced a decline in Ca levels. Interestingly, V1 displayed an increase in Ca after the infection, recording the highest post-infection value, while V3 had its lowest content (Fig. 6). The reductions in Ca levels due to the pathogen attack were -18.6% (V2), -56.2% (V3), -34.08% (V4), and -24.5% (V5).

Post-infection magnesium (Mg) levels also varied significantly ($p \leq 0.05$) across all the rice lines. Contrary to the control, the pathogen attack exerted considerable destruction in magnesium contents. Declining responses were seen in all the rice types after the infection, with V1 and V2 documented for the highest value of this attribute, whereas V4 showed the lowest concentration of Mg ions in the normal and infection condition. Overall, the pathogen attack caused maximum destruction in V2 and V4 (-31.6 and 40.8%, respectively).

4. DISCUSSION

The concept of the growth-defense tradeoff is reckoned as the basic dogma in survival under pathogen attack, allowing plants to modulate growth activities and defense responses according to prevailing environmental conditions (He *et al.*, 2022; Walters *et al.*, 2014; Bechtold *et al.*, 2018). Our results indicated a notable reduction in growth among rice plants highlighting varying levels of defense against *H. oryzae*. These findings suggest that such traits can help in identifying rice varieties as resistant or susceptible to pathogen attacks. The observed decline in growth reflects the damage caused by *H. oryzae* and the plant ability to mitigate that damage. Additionally, the decrease in root and shoot length due to the pathogen presence aligns with previous studies (Schwarz and Grosch, 2003; Grosch and Schwarz, 1998; Djalali Farahani-Kofoet *et al.*, 2020; Corpas-Hervias *et al.*, 2006; Ghaemi *et al.*, 2011). Highly susceptible plants often lose their ability to effectively fight the pathogen, resulting in poor growth and survival. Although overall growth loss was observed in all the rice lines, the relatively lesser reduction in fresh and dry biomass in V3 and V1 suggests their innate capacity to fight the pathogen. This resilience is attributed to the effective allocation of internal resources toward immune responses, molecular modulations, and growth activity. Although some reduction in growth was evident, the reallocation of resources to biochemical and physiological processes helped in protecting these plants. This resource reallocation and adjustment are regulated by a complex molecular machinery, *i.e*. transcription factors (TF), genes *etc*., which are key to enabling successful defense mechanisms. Previously, it was thought that this resource shifting resulted in a trade-off between growth and defense (Cai and Aharoni, 2022; Todesco *et al.*, 2010; Krol *et al.*, 2010). Our findings are in complete consensus with these observations. We also propose that the growth-defense tradeoff could stem from the differential activation of various pathways, including those associated with plant hormones and light response (Xu *et al.*, 2017; Bechtold *et al*., 2018). On the other hand, it has been established that primary metabolic pathways and defense responses are strongly associated. A molecular analysis of *Arabidopsis* under pathogen attack revealed a crucial role of multiple TFs and genes in maintaining metabolic processing and timely output to ensure appropriate defense response (Less *et al.*, 2011). An interesting facet of this relationship and coordination among molecular, physiological, and biochemical attributes is that the expression of genes for any attribute can be negatively, positively, or not correlated at all in response to stress or any stage of stress (Less *et al*., 2011). This supports our viewpoint that the growth-defense tradeoff is not merely a cost of growth but also involves antagonistic processes that play a role in preventing or protecting rice plants from the damage caused by pathogens. A failure in the coordination of these metabolic and defense adjustments likely results in poor growth and heightened susceptibility to infection (He *et al*., 2022, Bechtold *et al*., 2018).

The reduction in chlorophyll observed in this study is directly linked with reduction of other biochemical compounds, *e.g*. sugars and carotenoids (Shabana *et al.*, 2008; Dallagnol *et al.*, 2011). The progressive destruction of chlorophyll due to pathogen attack results in reduced light capture for photosynthesis that leads to poor plant growth, as observed in this study. The decrease in essential nutrients like Mg, Ca, and K is directly linked to the reduction in chlorophyll content. Besides, changes in ion balance may act as a stress signal triggering defense mechanisms against infections (Atim *et al.*, 2013; Gowtham *et al.*, 2024). The defense response of plants is intricately linked to calcium levels, as calcium ions play a central role in signal transduction processes. The reduced growth of plants particularly after *H. oryzae* attack is indicative of disturbance in tissue ionic homeostasis. Additionally, our stance gets support from the finding that plants respond to a variety of signals, including biotic and abiotic stressors, by managing their cytoplasmic Ca concentrations. These signals have been linked to the host plant's defense mechanism (Cruz *et al.*, 2012; Choi *et al.*, 2013). A decreased Ca level means less signaling and ultimately reduced defense to *H. oryzae.* On the other hand, it is well documented that up-regulated primary metabolism is coordinated with signal transduction cascades and culminates in plant defense response(s) (Rojas *et al.*, 2014). The synthesis or degradation of these biochemicals is directly associated with induction, regulation, or deregulation of many genes. For instance, the induction of *AtPR1* and *AtPR5* by glucose was dependent on *AtHXK1.* However, in *Nicotiana benthamiana*, silencing of *HXK1* increased H_2O_2 accumulation and its negative association with programmed cell death (Kim *et al.*, 2006).

These facts are in direct relationship with our recorded changes in growth and metabolism. The recorded changes in growth and losses due to infection particularly in terms of MDA and H_2O_2 are clear reflection of overall changes in plant metabolism, metabolic reprograming, and differential attenuation of immunity in rice plants. These insights provide a solid foundation for developing rice varieties with enhanced resistance to *H. oryzae*.

The hypothesis that *H. oryzae* infection induces oxidative stress through membrane-lipid peroxidation is supported by the observed increase in the MDA content. Lipid peroxidation leads to the synthesis of MDA, which in turn contributes to a decrease in antioxidant defenses (Munné-Bosch and Alegre, 2002; Ramzan *et al.*, 2021). This process may increase membrane fluidity, a consequence of microbial attack on the plant's cellular structures (Dallagnol *et al*., 2011; Singh *et al.*, 2013; Noman *et al.*, 2018b). The simultaneous reduction in ROS scavenging activity and increased ROS production may trigger programmed cell death or a hypersensitive response. Following pathogen infection, enzymatic antioxidant activities differed between susceptible and resistant rice plant (Mittler, 2017; Shamshad *et al*., 2024). Lower MDA levels, conversely, indicate reduced lipid peroxidation and less oxidative stress. Antioxidant enzymes such as SOD and POx play key roles in scavenging excess ROS. Superoxide dismutase is considered as the first line of defense and it converts O^{2-} to H_2O_2, aiding in stress tolerance, which is further converted to H_2O by CAT, APX, and GPX or through the AsA-GSH cycle. This cascade of antioxidant activities underscores their essential role in rice plant survival under pathogen attack (Ramzan *et al.*, 2021; Noman *et al.*, 2018a; del Río *et al.*, 2018; Shamshad *et al*., 2024). The drop in ascorbic acid levels noted in Binicol could very well be linked to its higher vulnerability because of various factors. Ascorbic acid, or vitamin C, is extremely important for supporting the antioxidant defense system of plants. It aids in mitigating the effects of reactive oxygen species (ROS) that are generated in stressful conditions, like an attack from a pathogen or an environmental stress. A considerable drop in the ascorbic acid concentration compromises the plant's capacity to deal with oxidative stress, thus increasing its chances of being damaged by pathogens or unfavorable environmental factors. Lowered ascorbic acid concentrations may therefore hinder the immune system of the plant, making it more prone to diseases or stress. Supporting this notion, Mofidnakhaei *et al.* (2016), Ramezani *et al.* (2017), and Mohammadi *et al.* (2019) unanimously reported that attacks by *Pseudoperonospora cubensis* and *Podosphora infestans* in potato and cucumber enhanced SOD, APX, and CAT activities. Akbar *et al.* (2023) identified two categories of transcripts in *Saccharum* sp.: i) ROS producing and ii) ROS scavenging genes (Akbar *et al.*, 2020). In total, 329 of 757 transcripts were involved in ROS production, while 428 were involved in ROS scavenging pathways (Akbar *et al*., 2020). This induced systemic resistance (ISR) response is known to provide broad-spectrum defense against a range of pathogens (Singhai *et al.*, 2011). Consistent with our findings, CAT and SOD activities in tomatoes were increased during the early phase of *B. cinerea* infection but decreased following the formation of necrotic lesions (Kużniak and Skłodowska, 2005). The variation in CAT activity across rice lines suggests its key role in resistance to brown leaf spot. Significant differences in APX activity in *H. oryzae*-challenged rice highlight its importance in the collective defense response. Comparable modifications in POD, APX, and GST were also reported in barley after pathogen infection, which reinforced the importance of these antioxidants in plant resistance (Harrach *et al.*, 2008). Increased PPO and PAL activities were observed across all the rice lines with higher enzyme levels linked to greater resistance, highlighting their role in plant defense. Similar findings reported by Ngadze *et al.* (2012) showed that resistant potato varieties exhibited simultaneous increases in PPO and PAL activities, along with changes in metabolites like total phenolics, which play a critical role in stress tolerance (Ngadze *et al.*, 2012). This suggests a metabolic link between PPO activity and phenolic compounds contributing to biotic stress resistance such as soft rot tolerance. The observed variations in growth traits, chlorophyll content, antioxidant activity, and secondary metabolite levels among the rice lines provide valuable insights for identifying pathogen resistance traits. Notably, the resilience of V3 characterized by superior growth, enhanced antioxidant enzyme activity, and effective metabolic adjustments under *H. oryzae* stress positions it as a strong candidate for breeding programs targeting improved disease resistance.

Phenolic accumulation is generally associated with the production of other metabolites, such as lignin deposition, pathogenesis-related (PR) proteins, and enzymes (Myers *et al.*, 2000; Talieva and Kondrat'eva, 2002). Similar to total phenolics, variations in lignin, flavonoids, and anthocyanins were recorded in the pathogen-infected rice plants. Reduced lignin content raises concerns about increasing disease susceptibility (Dang *et al.*, 2014; Mou *et al.*, 2013; Myers *et al.*, 2000; Talieva and Kondrat'eva, 2002). Variations in lignin, flavonoids, and anthocyanins were noted in the pathogen-infected rice, with reduced lignin content raising concerns about increased disease susceptibility. V5 showed a significant decrease in their lignin and flavonoid contents compared to the other rice plants, which could altogether alter immune responses to pathogens.

The reduced lignin content in V5 likely reflects its inability to deposit lignin adequately, resulting in increased vulnerability to *H. oryzae*. Recent literature offered a new insight by clarifying that reduction in lignin levels in alfalfa (*Medicago sativa* L.) is linked to higher accessibility of cell wall polysaccharides within plant cells (Bhuiyan *et al.*, 2009). Additionally, plant cell wall lignin buildup is frequently linked to pathogen invasion and is thought to be

the initial stage in preventing pathogen invasion (Gill *et al.*, 2018). Similar results were observed in other plants after mutation of the lignin production gene. The evidence to support our stance comes from fruits. Owing to their high flavonoid content and low pathogen prevalence, unripe fruits are typically more resistant to fungal deterioration (Treutter, 2006).

5. CONCLUSIONS

This study revealed significant variation among rice lines in their physiological, biochemical, and ionomic responses to *Helminthosporium oryzae* infection. By exhibiting superior growth attributes, higher antioxidant enzyme activities, and minimal reductions in chlorophyll and root biomass, Kharamana consistently outperformed the other varieties, positioning it as the most resilient line. Particularly the ability of Kharamana to maintain potassium losses under pathogen stress highlights its robust ionic homeostasis. These findings emphasize the potential of V3 for breeding programs aimed at enhancing rice resilience against *H. oryzae*-induced stress. The superior resilience of Kharamana against *H. oryzae* highlights its potential for use in breeding programs aimed at developing rice varieties with enhanced stress tolerance, contributing to sustainable rice production and food security. Unveiling the genetic and molecular mechanisms behind the resilience of V3 against *H. oryzae* could facilitate transgenic and genome-editing strategies to enhance rice resistance. Future research on immune pathways and the rice microbiome can further improve crop growth and stress tolerance.

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Conflict of Interest. Authors declare no conflict of interest.

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