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Evaluation of biochemical traits and gene expression in wild and mutant rice cultivars under salt stress

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ABSTRACT

Salinity stress is one of the most significant abiotic challenges affecting crop production worldwide, posing a serious threat to agricultural yields globally. Rice, which ranks second in global production after wheat, is particularly sensitive to salt stress. Developing salinity-tolerant rice varieties is crucial for mitigating yield reductions in saline environments. Therefore, investigating and identifying the expression patterns of effective genes in response to salinity stress is essential for introducing tolerant genotypes. In this study, we examined an advanced mutant line of Hashemi rice (tolerant to salinity stress) resulting from gamma-ray irradiation, alongside its wild counterpart (Hashmi line - sensitive to salt stress). Both lines were subjected to salt stress in randomized complete block design with three replications using a hydroponic solution. The main factors in the factorial design included salinity treatment (0, 100, and 150 mM sodium chloride) and sampling time, with genotypes as the sub-factor. We evaluated biochemical traits and gene expression post-salt stress through qRT-PCR analysis. The results indicate significant effects of salinity stress on biochemical traits and gene expression in both wild and mutant rice cultivars. The mutants showed lower sensitivity to salinity compared to the wild cultivar, as evidenced by changes in biochemical traits, including chlorophyll content, antioxidant capacity, and sodium and potassium ion concentrations. Additionally, gene expression analysis revealed that several salinity tolerance-associated genes, such as superoxide dismutase and catalase, were expressed at higher levels in the mutants than in the wild cultivar. These findings suggest that different mechanisms are involved in the response to salinity stress between the wild and mutant cultivars, which could inform breeding strategies aimed at enhancing salt tolerance in rice. Consequently, this study lays the groundwork for future research into identifying and analyzing salt tolerance genes in rice and other agricultural crops, ultimately aiming to develop effective strategies to mitigate environmental stresses.

***Key words*:** Antioxidant genes, Mutation, Rice, Salinity.

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INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important agricultural crops, primarily composed of carbohydrates in the form of starch, which constitutes 50 to 90% of the grain's dry weight. Additionally, it contains 5 to 12% protein and 1 to 3% fat (Rodríguez Coca *et al*., 2023). Rice provides food for more than one-third of the world's population (Chauhan *et al*., 2017). Food security has become a critical global issue due to increasing population growth and urbanization (Wang *et al*., 2019). Consequently, food shortages, malnutrition, and ensuring the well-being of millions of consumers are pressing concerns (Stevens *et al*., 2022).

Salinity is a primary factor that limits the growth and development of rice, and despite the development of salt-tolerant varieties identified at different growth stages, rice remains classified as a salt-sensitive plant (Zhang *et al*., 2019; Chen *et al*., 2021). Biotic and abiotic stresses significantly restrict rice growth and yield. It is projected that by 2050, over 50% of the world's agricultural lands will experience drought and salinity stress (Chauhan *et al*., 2017). Currently, half of Iran's arable land (9.5 million hectares) is affected by salinity, leading to a considerable reduction in cultivated area and crop yield (Nabiollahi *et al*., 2017). The increase and accumulation of various types of salts in different soil layers have affected a substantial portion of the world's arable land. Reports indicate that approximately 33% of irrigated lands and 20% of all arable lands globally are impacted by soil salinization processes (Al-Tawaha *et al*., 2021).

Plants can adapt to high levels of salinity stress through two primary mechanisms: preventing intracellular salt accumulation or sequestering it within the cell vacuole. Both processes result in maintaining a relatively low concentration of cytoplasmic salt (Kamrava *et al*., 2024). Furthermore, it is estimated that by 2050, more than 50% of the world's agricultural lands will be exposed to salinity stress, particularly in fertile regions like the Mediterranean and South Asia (Alam *et al*., 2021). Salinity stress is a significant abiotic factor that hinders the growth and development of crops globally (Ganie *et al*., 2021). During salt stress, rice activates various defense responses, including morphological, ultrastructural, and physiological changes in plant organs, as well as biochemical productions and the initiation of molecular activities (Qin *et al*., 2020). Although rice is a crucial staple food in developing countries, its cultivation is often severely affected by salinity stress (Razzaq *et al*., 2020). The roots are the first part of the plant exposed to soil salinity; damage to the root system consequently reduces the growth of aerial organs by decreasing leaf surface area. However, studies indicate that aerial parts are more sensitive to salinity than roots (Pour-Aboughadareh *et al*., 2021; Rasel *et al*., 2021).

Salt stress affects the morpho-physiological and biochemical characteristics of tissues and cells, ultimately leading to plant diseases and significant yield loss (Gupta and Huang, 2014). When the balance between the production of active oxygen and the required amount for the antioxidant defense system is disrupted, excessive production and accumulation of reactive oxygen species (ROS) occur, leading to oxidative stress in plants. This oxidative stress is one of the critical consequences of salinity (Saleem *et al*., 2020).

One of the most effective strategies for mitigating yield reductions in saline environments is the development of salt-tolerant varieties. Further investigation into the physiological and genetic mechanisms related to salt tolerance is crucial for addressing this global issue (Zeeshan *et al*., 2020). Inducing mutations through mutagens is a method to create genetic diversity (Okamura *et al*., 2012). Lin *et al*. (2016) reported that utilizing salt-sensitive mutant lines is a valuable tool for identifying genes involved in salt stress. A mutant population generated and studied using sodium azide mutagenesis in rice resulted in the identification of a hypersensitive mutant (Abdelaziz *et al*., 2018).

The main objective of this study is to evaluate and analyze biochemical and enzymatic traits, as well as investigate changes in gene expression related to salt tolerance in both wild and mutant rice cultivars. Specifically, this research aims to identify biological mechanisms that effectively enhance tolerance to salinity stress and to provide strategies for increasing plant resistance to this environmental challenge, ultimately improving rice health and crop production.

MATERIALS AND METHODS

Cultivation, application of stress, and sampling

In this study, Hashemi genotype rice seeds were used as the susceptible wild type, while advanced mutant lines (seventh generation) were obtained from the Rice Research Institute of Kashgar-Rasht. The mutant line was created through gamma irradiation from a cobalt-60 source and was introduced as a salt-tolerant line in field evaluations, which identified it as tolerant

to salinity levels of 8 dS. The present study was conducted in a randomized complete block design with three replications and three levels of salinity stress (0, 100, and 150 mM sodium chloride) using a hydroponic solution.

Seeds were sterilized with a 10% calcium hypochlorite solution and placed in a dark incubator at 23 °C for six days for germination. Identically germinated seeds were then transferred to Yoshida culture medium (Yoshida *et al*., 1976) (Figure 1). The nutrient solution was replaced every three days, and the pH was adjusted to 7.5 with sodium hydroxide (NaOH). After six days of growth in the normal culture medium, salinity stress was applied by adding 100 and 150 mM sodium chloride for six days. The first sampling was conducted three days after the application of salinity stress, and the second sampling was performed six days post-application, using both normal and saline media from root tissue. Samples were immediately frozen in liquid nitrogen and stored at -80 °C for biochemical measurements.

Figure 1. View of rice genotypes planted in a hydroponic culture environment.

Measurement of biochemical and enzymatic traits

Enzyme activity is expressed in units per milligram of total protein extracted. This unit indicates the amount of enzyme activity present per milligram of protein. Superoxide dismutase activity was determined following the method described by Beyer and Fridovich (1987), while catalase activity was measured according to Aebi *et al*. (1984). Ascorbate peroxidase enzyme activity was assessed based on the method outlined by Nakano and Asada (1981), and glutathione reductase enzyme activity was determined using the procedure described by Smith (1989).

RNA extraction, cDNA synthesis, and gene expression assessment

RNA was extracted using the P-Biosol extraction buffer from Bioflex (Tokyo, Japan), and its quality was assessed by electrophoresis on a 1.5% agarose gel. Subsequently, cDNA synthesis was carried out according to the protocol provided by Fermentas (Canada), and the synthesized cDNA was tested using PCR with Actin housekeeping gene primers, as described by Kazemi *et al*. (2010).

To assess the expression patterns of catalase, superoxide dismutase, glutathione reductase, and ascorbate peroxidase genes, the iQ5 device from Bio-Rad and the Cyber Biopars kit from Gorgan University of Agricultural Sciences and Natural Resources were utilized. The Cyber Biopars kit allows for real-time evaluation of gene expression. To normalize the data, the housekeeping gene Actin, which maintains consistent expression across all treatments, was employed. Primers were designed using information from the NCBI website and Primer 3 software, ensuring specific characteristics for qRT-PCR. Product sequences ranged from 132 to 187 bp, with melting temperatures between 51.4 and 60 °C determined by GC percentage and band length (Table 1). Following the completion of the reaction and obtaining the melting curve diagram (Figure 2), data were analyzed using REST software (Moloudi *et al*., 2013).

RESULTS AND DISCUSSION

The results of the analysis of variance indicated that the triple interaction of time, stress, and genotype was significant at the 1% level for all evaluated traits (Table 2).

Superoxide dismutase enzyme activity

Superoxide dismutase (SOD) enzyme activity increased with rising salinity levels. The highest activity of this enzyme in the mutant genotype was observed three days after salt stress, surpassing the activity observed in the wild genotype (Figure 2). In the second sampling, the greatest enzyme activity was recorded in the treatment of 150 mM salt in the mutant genotype, demonstrating a significant difference from the first sampling conditions. Antioxidants are molecules that prevent the oxidation of other molecules, and plants utilize both enzymatic and non-enzymatic antioxidant mechanisms to combat oxidative stress induced by salt stress. The antioxidant enzyme superoxide dismutase is recognized as one of the fastest defenses against the attack of active oxygen (Fakhrfeshani *et al*., 2024). The level of antioxidant activity determines the extent of oxidative stress and is positively correlated with salt stress tolerance levels (Kaya *et al*., 2018). Superoxide dismutase is considered the most critical antioxidant enzyme, capable of effectively protecting plants against reactive oxygen species (ROS) attacks and enhancing

Table 1. Specifications of the primers used.

Initiator name	Primer sequence	Product length	Melting temperature (°C)	NCBI accession number
CAT1 FOR *CAT1* REV	5′- TCATCTCTTGTTAATTAATTGGAGTACTAC-3′ 5′- GAAGTGATAATTTAAATACTTAATAGTAAT-3′	206	60 57.9	AK099923
Actin FOR *Actin* REV	5′-TCCCGAGTATTGTTGGTCGT-3′ 5′-TCCATGTCATCCCAGTTGCT-3′	176	58 58	AF111812
APX1 FOR *APX1* REV	5′- TAGTCTACTACTGCTAGTAC-3′ 5′- TAACAGCCCACCGAGACATT-3′	207	60 60	AY382617
GR FOR *GR* REV	5′-TGTTGACTGAGACTACGGCC-3′ 5′-AGCTCCAATCACTCCCACAG-3′	195	60 60	XM_015770519
SOD1 FOR *SOD1* REV	5'-GTCACCGCTGGAGAAGATGG-3' 5'-GATGATCCCGCAAGCAACAC-3'	189	58 60	KU179440.1

Table 2. Variance of root enzyme traits of wild and mutant rice genotypes under salt stress conditions.

Sources of variation	df	Glutathione reductase	Catalase	Ascorbate peroxidase	Superoxide dismutase
Block	2	0.0001^{ns}	0.47^{ns}	0.0004^{ns}	0.001^{ns}
Stress	2	856.81**	707.96**	0.28**	277.44^{**}
Time	1	125.58**	0.9^{ns}	0.08**	10.14**
Stress×time	2	6.61**	96.6**	0.004*	0.09^{ns}
Error 1	10	1.93	3.45	0.001	0.72
Genotype	1	911.43**	347.7**	0.36**	24.37**
Genotype×stress	2	55.81**	55.35**	0.25**	9.43**
Time×genotype	1	28.38**	10.33**	0.06**	35.28**
Genotype×stress×time	2	6.33**	7.92**	0.048**	16.33**
Error 2	12	1.26	2.36	0.0006	0.38
Coefficient of variation (%)	5.09	2.31	1.80	2.31	6.46

ns, *, and ** indicate non-significance, significance at the 5% and 1% level, respectively.

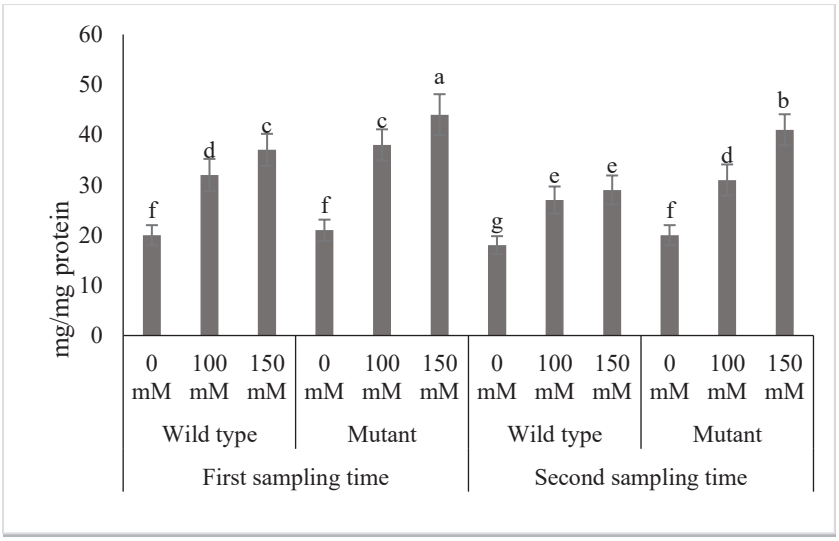

Figure 2. Superoxide dismutase levels in wild and mutant rice genotypes under salt stress (LSD test, p≤0.05). Means with the same letter are not significantly different.

plant stability under stress (Gill and Tuteja, 2010). This enzyme is responsible for desmutating superoxide radicals and converting them into hydrogen peroxide (Qiao *et al*., 2020). Reports in *Arabidopsis* (Shafi *et al*., 2015) and wheat (Wang *et al*., 2016) have demonstrated that increased expression of superoxide dismutase enhances tolerance to salt stress. The elevated activity of superoxide dismutase in response to salt stress reflects the plant's effort to manage the oxidative stress resulting from free radical production. This enzyme is crucial in minimizing cell damage by converting superoxide radicals into hydrogen peroxide, and the findings suggest that increased SOD activity could enhance the plant's ability to withstand salt stress.

Catalase enzyme activity

The results for catalase enzyme activity indicated that the mutant genotype exhibited higher levels of catalase activity in both normal and saline (150 mM) conditions compared to the wild genotype, with a statistically significant difference noted under salt stress conditions (Figure 3). The increase in catalase activity in the mutant genotype under normal conditions may be attributed to the effects of the mutation. One potential reason for the decrease in catalase activity observed six days after salt stress, compared to three days after application in the roots, could be the presence of catalase inhibitory agents such as salicylic acid (Jayakannan *et al*., 2015). An increase in catalase activity during salinity stress has been documented in various plant species, including rice (Abdelaziz *et al*., 2018), barley (Kiani *et al*., 2017), and wheat (Askari Kolestani *et al*., 2016). Catalase, which is present in plants and other aerobic organisms, breaks down hydrogen peroxide (H_2O_2) produced by superoxide dismutase (SOD) into harmless byproducts, including water and oxygen (Sharma *et al*., 2012). Increased activity of the mutant catalase genotype was observed when exposed to 100 mM sodium chloride, indicating its superior ability to inhibit reactive oxygen species (ROS). Furthermore, elevated expression of catalase during salinity stress has also been reported in different plant species, such as *Cucumis sativus* (Zhou *et al*., 2018).

Ascorbate peroxidase enzyme activity

The results for ascorbate peroxidase enzyme activity indicated that the activity of this enzyme in the mutant genotype increased with the duration of salt stress, peaking on the third and sixth day after exposure to 100 and 150 mM salt stress (Figure 4). A statistically significant difference was observed in the antioxidant enzyme activity between the mutant and wild genotypes. The levels of hydrogen peroxide in the mutant genotype significantly increased compared to the wild genotype, with the highest levels observed on the second sampling day, particularly in the 150 mM treatment. This enhancement in antioxidant enzyme activity was especially effective in detoxifying hydrogen peroxide during salinity stress. Ascorbate is the most abundant antioxidant in plants and acts as an electron donor for various vital reactions, such as facilitating the Mehler cycle (Foyer and Noctor, 2011). Ascorbate peroxidase reduces hydrogen peroxide, and its activity is positively correlated with tolerance to oxidative stress (Correa-Aragunde, 2013). Numerous

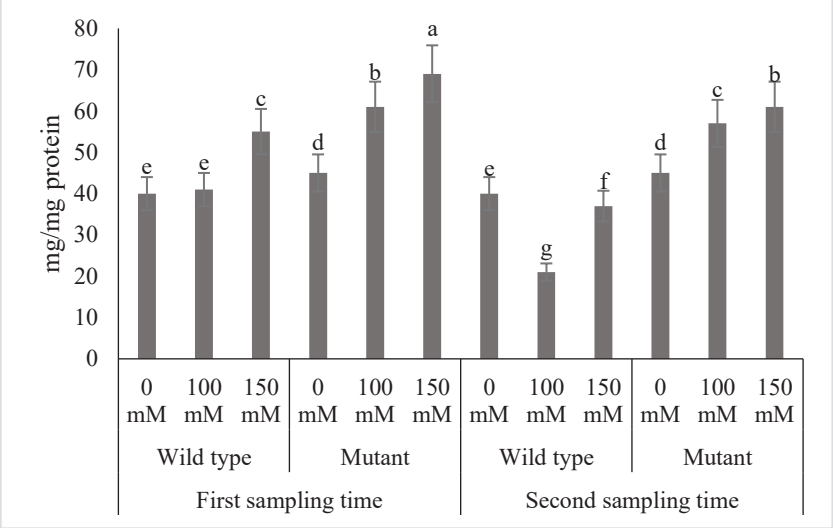

Figure 3. Catalase levels in wild and mutant rice genotypes under salt stress (LSD test, p≤0.05). Means with the same letter are not significantly different.

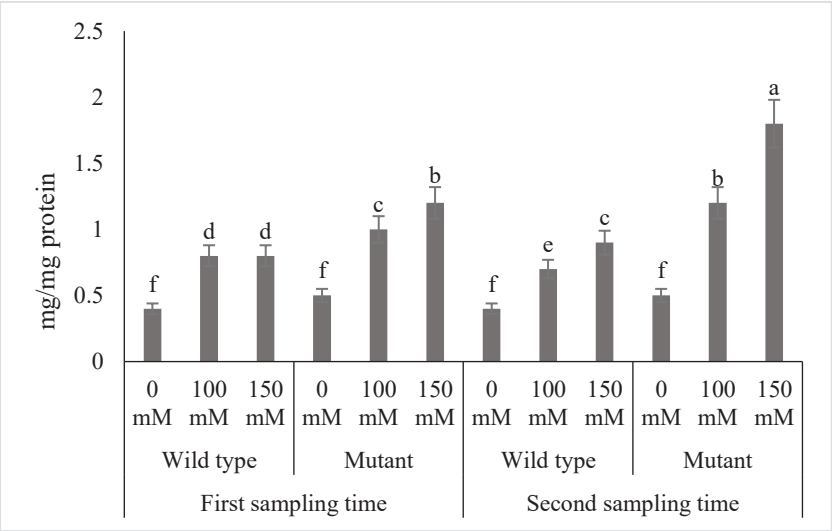

Figure 4. Ascorbate peroxidase levels in wild and mutant rice genotypes under salt stress (LSD test, p≤0.05). Means with the same letter are not significantly different.

studies have shown that increasing antioxidant enzyme activity correlates with reduced oxidative damage and enhanced salinity tolerance (Banu *et al*., 2010). In a study on the response to salinity stress in barley mutant genotypes compared to wild types, increased activity of the antioxidant enzymes catalase, superoxide dismutase, and peroxidase led to a significant decrease in hydrogen peroxide levels in the mutant genotype under salinity stress, contributing to greater tolerance (Kiani *et al*., 2017).

Glutathione reductase enzyme activity

Statistical analysis revealed a significant difference in the activity of glutathione reductase between the wild and mutant genotypes under both normal and stress conditions. The activity of this enzyme in the mutant genotype significantly increased compared to the wild genotype during the second sampling, specifically six days after salinity stress at 100 and 150 mM. This suggests a more effective response to salinity in the mutant genotype (Figure 5). The role of glutathione reductase in eliminating reactive oxygen species in the Halliwell/Asada pathway is well-established in plant cells. Glutathione reductase, within the ascorbate-glutathione pathway, is crucial for maintaining a high ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), which is essential for ascorbate production. Elevated levels of GSH are vital for enhancing tolerance to oxidative stress (Meloni *et al*., 2003). In a study of alfalfa, Ashrafi *et al*. (2015) observed that the salt-tolerant cultivar exhibited higher levels of glutathione reductase compared to the wild type in response to salt stress, indicating its role in reducing oxidative damage. In a proposed model for a rice mutant, the activity of glutathione reductase was found to be lower relative to lipid peroxidation levels. The products of lipid peroxidation can spontaneously bond with glutathione and diminish the activity of enzymes, including glutathione reductase and glutathione S-transferase. In this research, a correlation between elevated lipid peroxidation levels and decreased glutathione reductase activity was observed (Hazman *et al*., 2015). In response to salinity stress, plant antioxidant systems, particularly glutathione reductase, play a critical role in counteracting oxidative stress. Increased activity of glutathione reductase may enhance plant tolerance to salinity.

Superoxide dismutase gene expression

The expression of the superoxide dismutase (SOD) gene during salt stress in the two varieties of Hashemi rice increased with rising stress intensity at both sampling stages. The highest gene expression under the 150 mM salt stress treatment was observed in the mutant variety during the second sampling, showing a significant difference from other treatments (Figure 6). Salinity tolerance in rice is influenced by several genes; many antioxidant genes, including SOD, catalase (CAT), and ascorbate peroxidase (APX), as well as various de novo genes, are reported to be induced by salt stress (Ghosh *et al*., 2016). Superoxide dismutases represent the first line of enzymatic defense against oxidative stress (Qiao *et al*., 2020). In citrus fruits and tomatoes (*Lycopersicon esculentum*), salt tolerance has been associated with the activity of SOD genes (Gill *et al*., 2015).

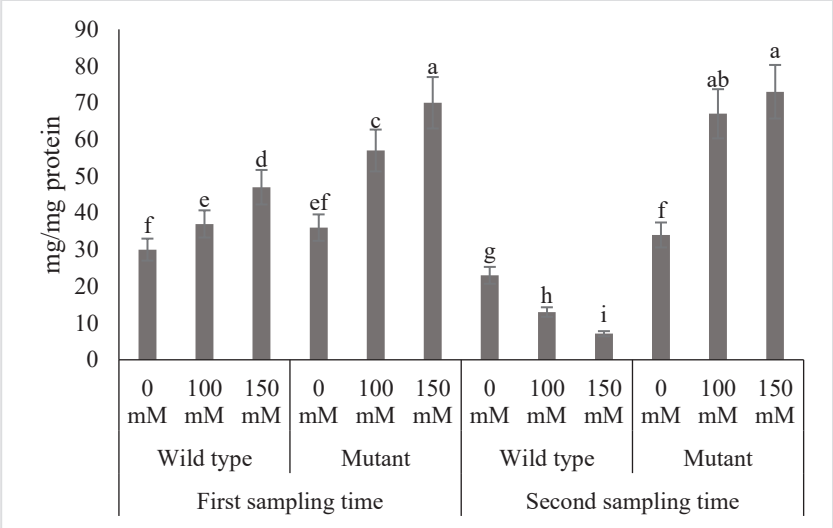

Figure 5. Glutathione reductase levels in wild and mutant rice genotypes under salt stress (LSD test, p≤0.05). Means with the same letter are not significantly different.

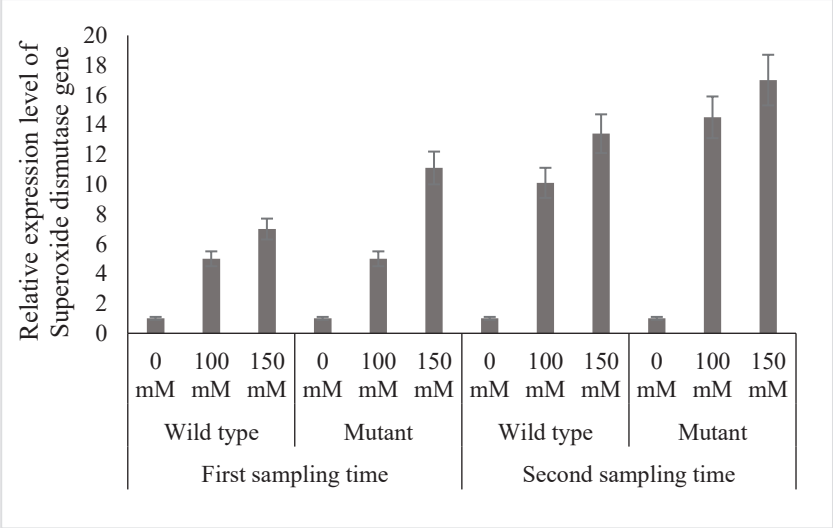

Figure 6. Relative expression of superoxide dismutase gene under different concentrations of salt stress in wild and mutant rice cultivars.

Catalase gene expression

The expression of the catalase gene increased in both genotypes during salt stress, correlating with the level of stress. The mutant variety exhibited the highest levels of gene expression during the 150 mM treatment in the first sampling, showing a significant difference compared to other treatments (Figure 7). Catalase (CAT) is remarkable for its high turnover rate, capable of exchanging 26 million molecules of H_2O_2 with one molecule of CAT in just one minute (Anjum *et al*., 2016; Kaushal *et al*., 2018). Several studies have indicated that the expression of catalase genes is temporally and spatially modulated in response to various environmental factors (Chen *et al*., 2012). In rice, three CAT genes (OsCatA, OsCatB, and OsCatC) are expressed under different environmental stresses and also contribute to modulating reactive oxygen species (ROS), photorespiration, and root development (Joo *et al*., 2014). Overexpression of OsCatA and OsCatC in rice has been shown to enhance tolerance to stress (Joo *et al*., 2014). Additionally, Vighi *et al*. (2016) reported that OsCatA and OsCatB are significantly affected by salinity stress, with their expression induced during cold stress in tolerant genotypes compared to sensitive ones. OsCatA exhibits predominantly higher expression levels throughout all growth stages in rice

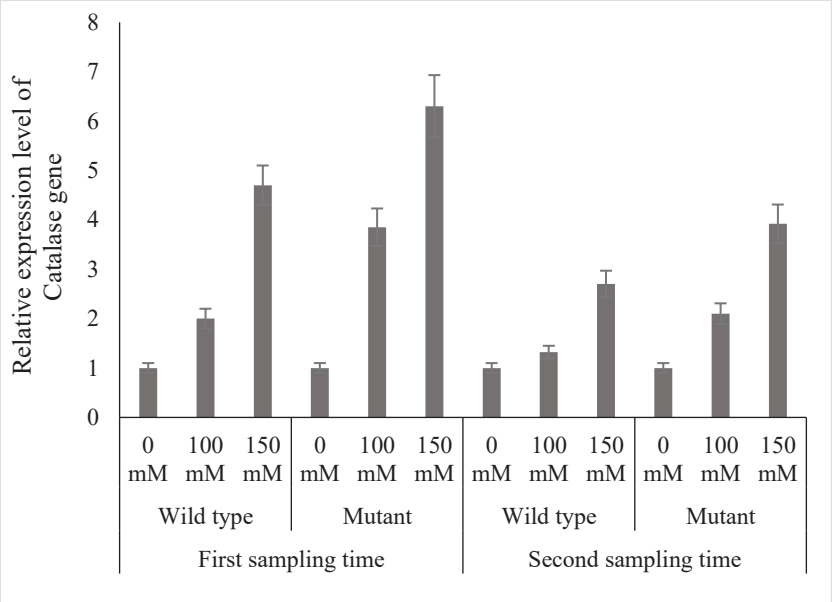

Figure 7. Relative expression of catalase gene under different concentrations of salt stress in wild and mutant rice cultivars.

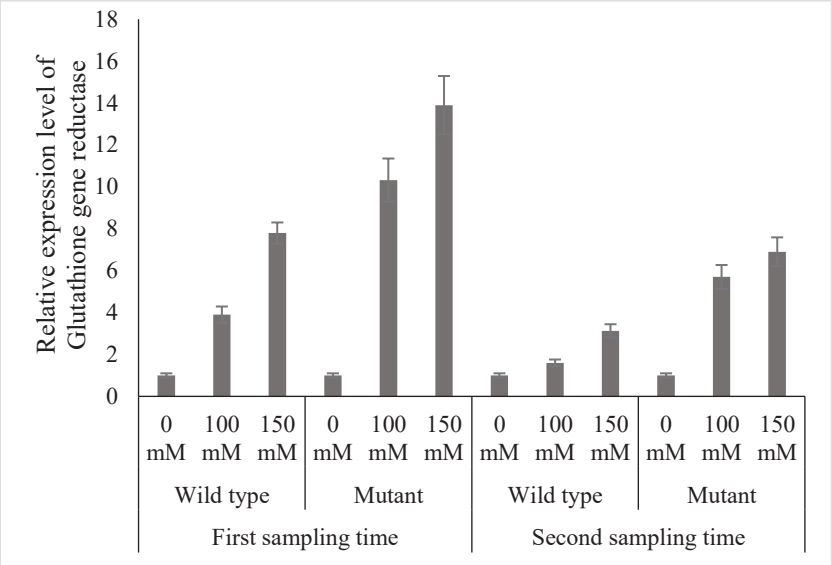

Figure 8. Relative expression of glutathione reductase gene under different concentrations of salt stress in wild and mutant rice cultivars.

(Alam *et al*., 2018). In cucumbers, the expression level of CsCAT1 is stimulated by ABA treatment, while CsCAT2 is repressed under drought stress, and CsCAT3 is upregulated following salinity, drought, and ABA treatment (Hu *et al*., 2016).

Glutathione reductase gene expression

The examination of glutathione reductase gene expression revealed that salt stress led to an increase in expression during the initial sampling stage. Notably, the mutant variety exhibited a more significant increase in gene expression compared to the wild variety. However, during the second stage of sampling, a decrease in gene expression was observed, although this decrease was less pronounced than that in the first stage. The highest level of glutathione reductase gene expression occurred during the first sampling, with a remarkable 13-fold increase compared to the control group in the mutant variety. This peak expression was observed at the 150 mM treatment (Figure 8). Glutathione reductase plays a crucial role in reducing hydrogen peroxide accumulation and maintaining

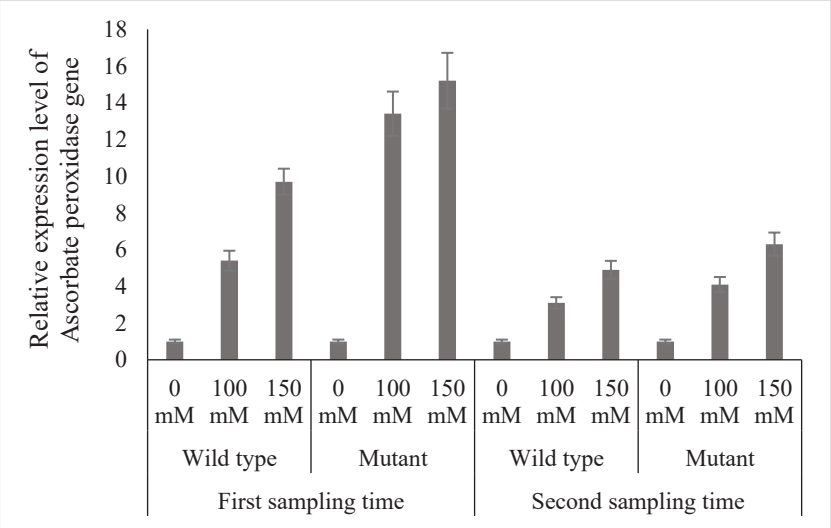

Figure 9. Relative expression of ascorbate peroxidase gene under different concentrations of salt stress in wild and mutant rice cultivars.

levels of reduced glutathione (Rahantaniaina *et al*., 2017). Glutathione exists in two states: reduced (GSH) and oxidized (GSSG). The ratio of reduced to oxidized glutathione within cells serves as an indicator of cellular oxidative stress, with a higher GSSG/GSH ratio signifying increased oxidative stress (Lu, 2013). Glutathione reductase helps decrease the GSSG/GSH ratio, regulating and supplying GSH for glutathione peroxidase (GPX) and dehydroascorbate reductase (DHAR), which convert H_2O_2 to water and reduce oxidized ascorbate, respectively (Bhabak and Mugesh, 2010). Research indicates that oxidative stress, pathogens, metals, cold, drought, and salinity can all increase the transcription of glutathione genes (Gao *et al*., 2016). For instance, elevated hydrogen peroxide levels and cold stress have been shown to enhance glutathione gene expression in rice (Passaia *et al*., 2013). Additionally, Lu *et al*. (2013) demonstrated that in wheat, glutathione reductase levels increased under stress conditions, a finding also observed in transgenic tobacco plants, where increased expression of the glutathione reductase gene enhanced salt stress tolerance.

Ascorbate peroxidase gene expression

The expression level of the ascorbate peroxidase (APX) gene increased with rising stress levels in both cultivars, reaching its highest value in the mutant cultivar under the 150 mM treatment, showing a 15-fold increase compared to the control. This increase was significantly greater than that observed in the other treatments (Figure 9). Ascorbic acid (AsA) serves as the first line of defense in plants against oxidative stress by eliminating several free radicals, including superoxide (O_2^-), hydroxyl radicals (HO·), and hydrogen peroxide (H_2O_2). This function is primarily mediated by APX, a key enzyme in the ascorbate-glutathione pathway (Bilska *et al*., 2019). Ascorbate acts as a cofactor for several cellular enzymes, including violaxanthin d-epoxidase, which is crucial for light protection through the xanthophyll cycle. It directly contributes to the removal of reactive oxygen species (ROS). The exogenous application of ascorbic acid has been shown to inhibit lipid peroxidation and reduce malondialdehyde (MDA) content in plant tissues, thereby increasing the antioxidant capacity of these tissues (Zou *et al*., 2016). To combat ROS, plants induce a variety of antioxidant genes, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), all of which help to neutralize ROS (Hameed *et al*., 2021).

CONCLUSION

The results of this experiment confirm that soil salinity significantly adversely affects growth and induces the production of reactive oxygen species (ROS) while also impacting their elimination within the cell. However, the plant possesses a robust defense system capable of mitigating ROS production. Further studies should be conducted to investigate the potential molecular mechanisms in cereal crops under saline conditions. The findings indicated that the mutant under study exhibited considerably greater inhibition of hydrogen peroxide by enhancing the activity of the antioxidant enzyme system at saline levels compared

to its wild counterpart. Consequently, the mutant line had the lowest levels of hydrogen peroxide and lipid peroxidation. This suggests that the mutant demonstrated resistance to salinity by augmenting the activity of antioxidant enzymes relative to the wild type. In contrast, the wild genotype displayed limited inhibitory systems, making it less capable of coping with salinity stress due to an inefficient antioxidant defense system. These results imply that the increase in antioxidant enzyme activity induced by salinity is crucial for protection against ROS. Therefore, exploring the effects of salinity stress through these enzymes can help identify resilient plant rootstocks, as there is a strong correlation between tolerance to environmental stresses and changes in enzyme concentrations in plants. Given that gene control regulates the production of substances within cells, identifying the genes responsible for producing these protective substances and transferring them to other plants could expedite the development of salt-resistant varieties. Considering the mutant genotype's superiority in most of the studied traits, it can be inferred that mutation is a valuable tool in plant breeding programs, offering heritable diversity for selection.

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