

Salt and Heat Stress Trigger Morpho-Physiological Changes, Antioxidant Enzyme and Secondary Metabolites Gene Expression in Rice (*Oryza sativa* L.)

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ABSTRACT

Climate change significantly increases salt and heat stress in rice plants. This condition causes plants to activate antioxidant enzymes and produce secondary metabolites. This study aimed to determine the morpho-physiological changes and gene expression profiles of antioxidant enzymes and secondary metabolites. This study used a completely randomized design factorial. The first factor was local rice varieties (*IR64*, *Silaun*, and *Cigeulis*), and the second factor was stress treatments (control, NaCl 150 mM, 40°C, and NaCl 150 mM + 40°C). The results showed that multiple stress significantly affected the plant height, stem length, stem diameter, leaf area, root length, total main root, plant biomass, necrotic length, chlorophyll content, relative water content, and plant ROS production. Multiple stress could up-regulate the gene expression of antioxidant enzymes (*Mn-SOD*, *Cu/Zn SOD*, *Cytosolic APX*, *OsAPX1*, *CAT*, *OsCATA*, and *GPOD*) in rice after stress combination treatments and increase the secondary metabolites gene expression (*P5CS* and *GABA-T*) in all rice varieties. Still, the *OsNOMT* gene was only active in the *Cigeulis* variety.

1. Introduction

Rice is a staple food that has been widely cultivated in Asia (92%), such as China, India, Thailand, and Indonesia (Javaid *et al.* 2019). IPCC (Intergovernmental Panel on Climate Change) states global temperatures increased to 1.1°C in 2011-2020 (IPCC 2022). This phenomenon significantly impacts the stress combination, producing a unique response in plants, including rice (Zandalinas *et al.* 2021). Rice plants can respond to stress by forming secondary metabolites, reducing their growth, or undergoing severe cellular damage that can lead to plant death (Zhou *et al.* 2016).

Salt stress is one of the most critical stresses that affect plant development. Based on FAO (2021), more than 10% of cropland has been salt-affected, which can be prone to a serious threat to global food security.

Salinity stress can occur when soil or irrigated water contains higher salts, such as NaCl, MgSO₄, MgCO₃, and CaCO₃ (Dar *et al.* 2021). This condition will negatively affect plant development due to chlorophyll reduction (Jan *et al.* 2021). Meanwhile, climate change can also increase the global atmospheric temperature, thus elevating the water demand by plants and reducing the water availability in land areas. As a result, photosynthesis efficiency and respiration rate will increase to a maximum and fall extremely until the plant dies (FAO 2010). The combination of salt and heat stresses can influence ROS accumulation, which enhances chlorophyllase activity and degrades photosynthetic pigments (Jan *et al.* 2021). Rice is sensitive to salinity and heat stress during early seedling and reproductive stages (Jan *et al.* 2021; Dar *et al.* 2021; Kilasi *et al.* 2018). Local rice has a more adaptive mechanism to the local environmental conditions, which can become a potential source for several abiotic tolerances (Dar *et al.* 2021).

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Salt and heat stress are two of the most common environmental stressors in rice cultivation (Zhao *et al.* 2010). This stress combination can lead to increased reactive oxygen species (ROS) production, which causes oxidative damage in plants (Mittler 2006; Mishra *et al.* 2018). This condition will trigger the biosynthesis of the abscisic acid (ABA) hormones as the plant's initial defense response (Boudsocq and Laurie`re 2005; Schmidt *et al.* 2013). ABA is a plant hormone that adapts to many abiotic stresses efficiently (Hirayama and Shinozaki 2007). ABA mediates the stomatal closure to prevent water loss due to osmotic stress caused by the stress combination (Suzuki *et al.* 2016). ABA also plays a vital role in increasing the activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), which are required in ROS scavenging (Zhang *et al.* 2008).

Another response in rice due to salt and heat stress is the production of secondary metabolites, such as proline, GABA, and sakuranetin (Sita and Kumar 2020; Nahar *et al.* 2022). Proline acts as an osmoprotectant to reduce damage caused by osmotic stress (Mishra *et al.* 2018). The function of γ -aminobutyric acid (GABA) was to mitigate abiotic stress via several mechanisms, including high antioxidant activity, reduction of ROS accumulation, membrane stabilization, and energy metabolism maintenance through the TCA cycle (Sita and Kumar 2020). Meanwhile, sakuranetin is a phytoalexin produced by rice when infected with *Pyricularia oryzae*, induced by jasmonic acid, and exposed to abiotic stress such as UV radiation (Shimizu *et al.* 2012). Meanwhile, the response of rice to individual stress factors has been extensively studied, yet limited to the rice response to a combination of salt and heat stresses, particularly regarding the expression of genes involved in GABA and sakuranetin biosynthesis. Therefore, this study aimed to describe the morpho-physiological changes in rice plants during salt and heat stress combination and gene expression profiling for antioxidant enzymes (SOD, CAT, APX, and GPOD) and secondary metabolites (proline, GABA, and sakuranetin). This study used local rice varieties, namely *IR64*, *Silaun*, and *Cigeulis*. The local varieties used in this study, i.e., *Silaun* and *Cigeulis*, have defense mechanisms for salt and heat stress. Meanwhile, *IR64* was used as a moderately resistant and control variety. Previous studies reported the responses of rice plants to salt and heat stress combination (e.g., Rivero *et al.* 2014;

Suzuki *et al.* 2016; Nahar *et al.* 2021; Jan *et al.* 2021). In this study, we used Indonesian local varieties with diverse traits potentially more adaptive to abiotic stresses in the local environments.

2. Materials and Methods

2.1. Plant Materials and Stress Treatments

Previously, a preliminary study was performed to find rice cultivars tolerant to salt and heat stress (data not shown). We found that *Cigeulis* and *Silaun* had high tolerance levels during salt and heat stress conditions. The rice plants were cultivated until 19 days old (seedling stage) for morphological analysis, and the 14-day-old rice (seedling stage) was used for physiology and genetic analysis. The seedling phase used in this study was selected as this stage is very sensitive to salt and heat stress during the seedling stage (Kilasi *et al.* 2018; Dar *et al.* 2021), thus showing a morphological, physiological, and genetic response when exposed to salt and heat stress combination.

This study was conducted using a factorial, completely randomized design with three replications. The first factor was local upland rice varieties as stress-tolerant cultivars: *IR64* (Mackill and Khush 2018), *Silaun* and *Cigeulis*. These rice varieties were obtained from the Indonesian Centre for Rice Research (ICRR). The second factor was the stress treatments: Control, 150 mM NaCl, 40°C temperature, and 150 mM NaCl + 40°C. Salt stress treatment was referred to Jan *et al.* (2021), while heat stress treatment was referred to Akhter *et al.* (2019) with modifications for the stress duration. The stress combinations were carried out by adding 150 mM NaCl to a nutrition bath and then putting it in an oven for heat exposure at 40°C with the dark condition for 4 hours.

2.2. Observation of Plant Morphology

Plant height and stem length were measured using a ruler (centimeters). Plant height was measured from the tiller part, which has the longest leaf of the plant. Meanwhile, stem length was measured from the root's base to the stem's end, where the first leaf was attached. The stem diameter (Bakhshandeh *et al.* 2017), root length (Xu and Hong 2013), and necrotic leaf length (Jia *et al.* 2012) were measured using a digital caliper (millimeters). The leaf area was calculated by ImageJ software. The leaf selected for leaf area analysis was

the widest leaf from a rice plant. Using an analytical balance, plant biomass was measured by weighing the plant's fresh and dry weights (70°C for 3 × 24 hours).

2.3. Relative Water Content (RWC)

Leaf relative water content was measured after the plants were treated for 7 days (21 DAP). Three leaves from each plant were used to represent the three replications. Plant leaves were cut along ± 10 cm and weighed as the fresh weight (FW). Turgid weight (TW) was weighed by immersing the leaves in distilled water for 12 hours. The leaves were oven-dried at 70°C for 24 hours and weighed as the dried weight (DW). Relative water content was calculated using the formula from Rafique *et al.* (2019):

$$\text{RWC} = (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100$$

2.4. Total Chlorophyll Content

The 100 mg of whole rice plant leaves were crushed by adding 5 ml of 95% ethanol. The sample was then centrifuged at 12,000 rpm for 10 minutes. The supernatant solution was measured for its absorbance at 664 and 649 nm wavelengths (Lichtenthaler 1987). The chlorophyll content in leaves was calculated using the following formula:

$$\begin{aligned} \text{Chl. A} &= (13.36 \times \text{Abs. 664}) - (5.19 \times \text{Abs. 649}) \\ \text{Chl. B} &= (27.43 \times \text{Abs. 649}) - (8.12 \times \text{Abs. 664}) \\ \text{Total Chl.} &= (5.24 \times \text{Abs. 664}) + (22.24 \times \text{Abs. 649}) \end{aligned}$$

2.5. Accumulation of MDA and ROS Levels

The accumulation of MDA levels was identified to determine the degree of cell damage caused by oxidative stress. The MDA content was calculated by adding 5 mL of 0.1% trichloroacetic acid (TCA) solution to a rice leaf sample (100 mg). The sample was then transferred into a 1.5 ml tube and centrifuged at 12,000 rpm for 10 minutes at 4°C. The 4 ml of TBA (thiobarbituric acid) was added to 1 ml of the supernatant and incubated at 90°C for 30 minutes. Incubation was performed to prevent polyunsaturated fatty acid autoxidation after adding the TBA solution (Bergamo *et al.* 1998). The absorbance value of the sample was determined at 532 and 600 nm wavelengths using a UV-Vis spectrophotometer.

The H₂O₂ levels were analyzed by homogenizing the 100 mg of fresh leaf samples using 1 ml of 0.1% TCA. The sample was then centrifuged at 12,000 rpm for 15 minutes. The 0.5 ml of the supernatant was taken

and transferred to a 1.5 ml tube, then 0.5 ml of 10 mM phosphate buffer, 1 ml of 1M potassium iodide, and incubated at room temperature for 30 minutes. The H₂O₂ levels were measured at 390 nm absorbance wavelength.

2.6. Expression of Antioxidant Genes and Other Metabolites

The 100 mg of fresh rice leaves (stored at -20°C before analysis) were ground using liquid nitrogen. The gene expression analysis procedures followed the protocol of Haryadi *et al.* (2023), which contained RNA isolation, cDNA synthesis, and PCR. Total RNA was extracted following the procedure of Ribospin™ Plant Kit (GeneAll) procedure, and cDNA synthesis followed the ReverTra Ace® qPCR RT Master Mix (Toyobo). The reverse-transcript Polymerase Chain Reaction (RT-PCR) was performed with a total volume of 15 µL, following the GoTaq® Green Master Mix (Promega) procedure. The PCR composition contained RT Master Mix (Toyobo), cDNA, primers (forward and reverse), and water (ddH₂O). For PCR, the cycle contained 2 cycles. The first cycle was incubated at 37°C for 15 minutes and 50°C for 5 minutes. The second cycle was heated at 98°C for 5 minutes. The annealing temperature and primer sequence are shown in Table 1. The PCR analysis was performed using the sequence primers from the genes: *OsActin*, *Mn-SOD*, *Cu/Zn SOD*, *CAT*, *OsCATA*, *GPOD*, *Cytosolic APX*, *OsAPX1*, *OsNOMT GABA-T*, and *P5CS*. The amplified RT-PCR products were then electrophoresed in a 2% agarose gel stained with 5 µL Ethidium Bromide. The electrophoretic gel was placed on the UV-transilluminator. The DNA fragments were then documented and observed for the thickness level. In this study, we quantify the gene expression based on the band thickness of the cDNA sample.

2.7. Data Analysis

The data were analyzed with ANOVA (Analysis of variance). If the results differed significantly, a further analysis was carried out using Duncan's Multiple Range Test with a 95% confidence level. The software used in this data analysis was Microsoft Excel 2010. The data from the visualization of electrophoretic gel was analyzed descriptively through visual presentation.

Table 1. Gene-specific primers sequence

Gene	Primer sequence	Source	Annealing temp. (°C)
<i>OsNOMT</i>	Forward: 5' CTA GCC GGA TGC ATG AAA GT 3' Reverse: 5' TGC ACG TAT AGG CAC ACA CA 3'	Shimizu <i>et al.</i> (2012)	54.00
<i>OsActin</i>	Forward: 5' TCC ATC TTG GCA TCT CTC AG 3' Reverse: 5' GTA CCC GCA TCA GGC ATC TG 3'	Kim <i>et al.</i> (2018)	55.35
<i>CAT</i>	Forward: 5' CAT CTG GCT CTC CTA CTG GTC T 3' Reverse: 5' CAG GAG AAA CGT GTC TTC AGG T 3'	Kim <i>et al.</i> (2007)	54.45
<i>OsCATA</i>	Forward: 5' CGG ATA GAC AGG AGA GGT TCA 3' Reverse: 5' AAT CTT CAC CCC CAA CGA CT 3'	Kim <i>et al.</i> (2018)	54.55
<i>Mn-SOD</i>	Forward: 5' GGA AAC AAC TGC TAA CCA GGA C 3' Reverse: 5' GCA ATG TAC ACA AGG TCC AGA A 3'	Kim <i>et al.</i> (2018)	58.2
<i>GPOD</i>	Forward: 5' ACC GTG AGC GAG GAC TAC CT 3' Reverse: 5' AGC GTC AAG TGA GCC TTA GC 3'	Kim <i>et al.</i> (2007)	55.45
<i>Cytosolic APX</i>	Forward: 5' AGT ACA TTG CCC GTG GTA CTC T 3' Reverse: 5' CGC ATT TCA CAA CAC ATC T 3'	Kim <i>et al.</i> (2007)	54.3
<i>Cu/Zn SOD</i>	Forward: 5' CAA TGC TGA AGG TGT AGC TGA G 3' Reverse: 5' GCG AAA TCC ATG TGA AGC TGA G 3'	Kim <i>et al.</i> (2018)	57.15
<i>OsAPX1</i>	Forward: 5' CCA AGG GTT ACC ACC TA 3' Reverse: 5' CAA GGT CCC TCA AAA CCA GA 3'	Kim <i>et al.</i> (2018)	54.3
<i>GABA-T</i>	Forward: 5' CTG AAT TCG CAG ACA ACA AAT C 3' Reverse: 5' TTA TCA TCG TCA TCG TCG TCT C 3'	Kim <i>et al.</i> (2007)	53.35
<i>P5CS</i>	Forward: 5' CAA ATG CTC CTT TTA GCC TGT T 3' Reverse: 5' GCG TTG GTA CAC AAG TTC TCA G 3'	Kim <i>et al.</i> (2007)	54.95

3. Results

3.1. Effects of Combination of Salt and Heat Stresses on Rice Morphology

There are no interaction effects on the combination of salt and heat stresses on rice morphology (Figure 1). The combination of stress treatments was significantly different on all morphological parameters, namely plant height, stem length and diameter, leaf area, total main roots, root length, number of necrotic leaves, length of necrotic leaves, and biomass parameters. The organs used in the morphology parameters were the whole plant (leaf, stem, and root organ). The plant height of *IR64*, *Silaun*, and *Cigeulis* varieties was reduced by 56.07%, 68.97%, and 57.96% simultaneously due to the stress combination treatment (Figure 1 and 2).

Figure 2 also shows a significant difference between the stem length of the control and stress treatment plants. The stem length of the *IR64* variety with stress combination treatment was reduced by 37.17%, the *Silaun* variety was reduced by 43.97%, and *Cigeulis* was reduced by 44.22%. Based on the data in Figure 2, the *IR64* variety has the lowest reduction percentage compared to the other two varieties.

The stem diameter of the *IR64* variety with stress combination was reduced at 30.17%. The highest reduction in the *IR64* variety was obtained from the saline treatment at 44.02%. Meanwhile, the

stem diameter of the *Silaun* variety was reduced by 50.90%. The stem diameter of the *Cigeulis* variety was also reduced by 41.5%. Therefore, the *IR64* variety has the least reduction percentage of stem diameter compared to the other two varieties.

In stress combination treatments, the leaf area of the *IR64* variety was reduced by 49.32%, the *Silaun* variety was reduced by 80.59%, and the *Cigeulis* variety was reduced by 70.04%. Based on the calculation of leaf area reduction, the *Silaun* variety obtained the highest reduction of leaf area. In contrast, the *IR64* variety obtained the least reduction in leaf area compared to the other two varieties with the same treatment.

The total main root increase in the *IR64* variety with stress combination was 4.35%. This condition was also found in the *Silaun* variety at 10% and the *Cigeulis* variety at 64.28%. Based on the total main roots, the *Cigeulis* variety obtained the highest main root increase among the two other varieties.

Figure 2 shows the root length in the *IR64* variety after the stress combination at 23.53%. The root length in the *Silaun* variety with stress combination was 4.91%. The root length of the *Cigeulis* variety with the stress combination also increased at 16.10%. Based on the results of three varieties, the *IR64* variety has the longest root length between two other varieties.

The visual appearance of rice plants after stress exposure treatment can be seen in Figure 1. In Figure 2, the stress combination caused a significant reduction in plant height, stem length and diameter, and leaf area. Based on Figure 2 and Table 2, the *Cigeulis* variety was more tolerant due to its lower growth reduction value than the *Silaun* variety. In contrast, the *IR64* variety was used as a control plant.

Other morphological parameters are the number of necrotic leaves and the length of necrotic leaves. These parameters illustrate cell death on the leaf surface. Both parameters present a highly significant difference between the stress treatment and the control. In the number of necrotic leaves, the stress treatments had no significant difference with likely similar values, while the control treatment had no necrotic leaves.

The length of necrotic leaves with stress combination treatments significantly differed from the two individual stresses on the three varieties. The lengths of necrotic leaves in *IR64*, *Silaun*, and *Cigeulis* varieties were 14.6, 22.42, and 14.58 cm. Based on the following results in the three rice varieties (Figure 3), the *Cigeulis* variety had the least elongation of necrotic leaves. In contrast, the *Silaun* variety had the highest elongation of necrosis leaves.

In biomass parameters, a significant reduction is presented in Figure 4. The *IR64* variety obtained a reduced fresh weight of 67.46%, while the dry weight was reduced by 41.11%. The *Silaun* variety fresh and dry weights were also reduced by 74.87% and 45.66%, respectively. The fresh weight reduction in the *Cigeulis* variety was 31.23%, while the dry weight was reduced by 15.13%. This biomass reduction could affect plant productivity since biomass is the amount of matter stored in plants. Based on the plant biomass reduction, the *Cigeulis* variety provides the lowest reduction in biomass under the same stress treatment, while the *Silaun* variety provides the highest reduction in biomass.

3.2. Combination Effects of Salt and Heat Stresses in the Rice Physiology

The observed physiological parameters were chlorophyll a, b, total chlorophyll, RWC, MDA, and ROS levels. The major rice organ used in the physiology parameters was leaves. Meanwhile, other organs, such as the root and stem, were used to observe

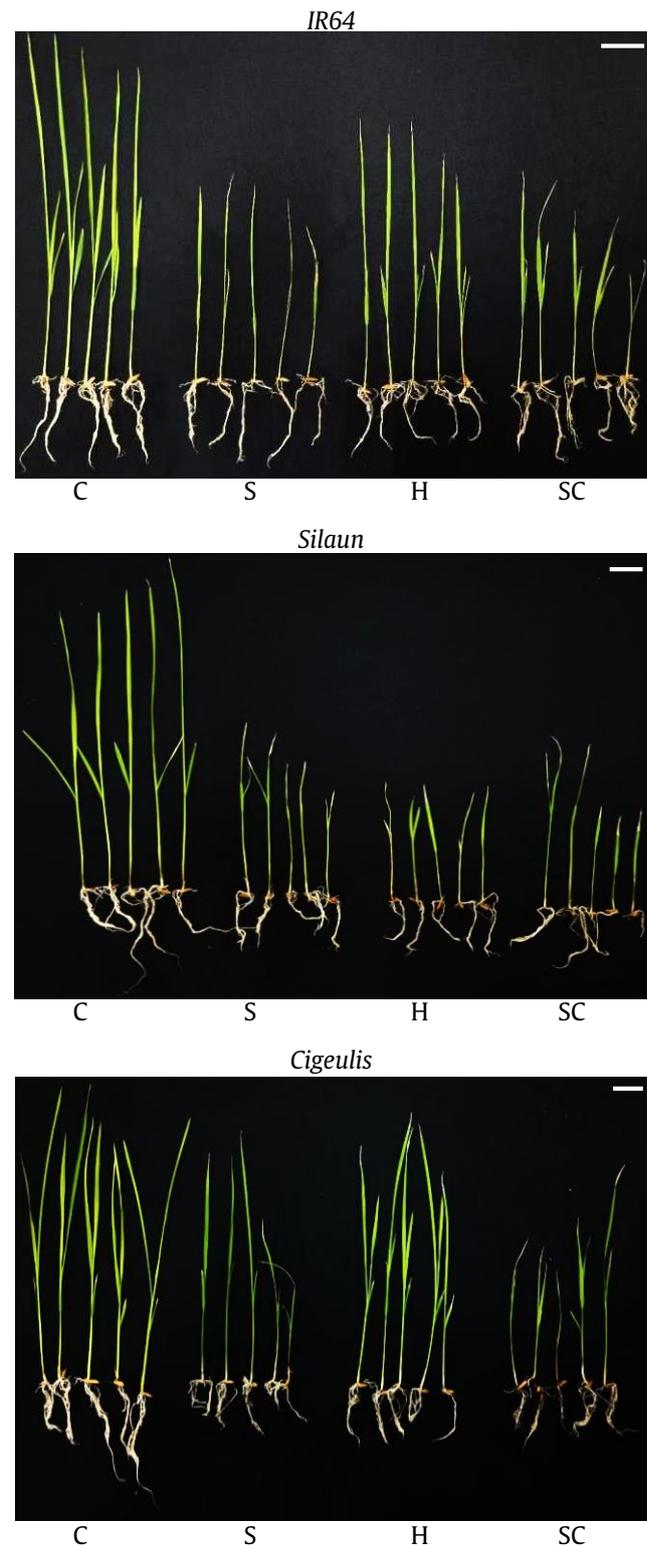


Figure 1. Rice seedling (19-day-old plant) of *IR64*, *Silaun*, and *Cigeulis* varieties after stress exposure treatments (scale bar = 2 cm). The plant treatments: Control (C), salt stress (S), heat stress (H), and stress combination (SC) for 5 days

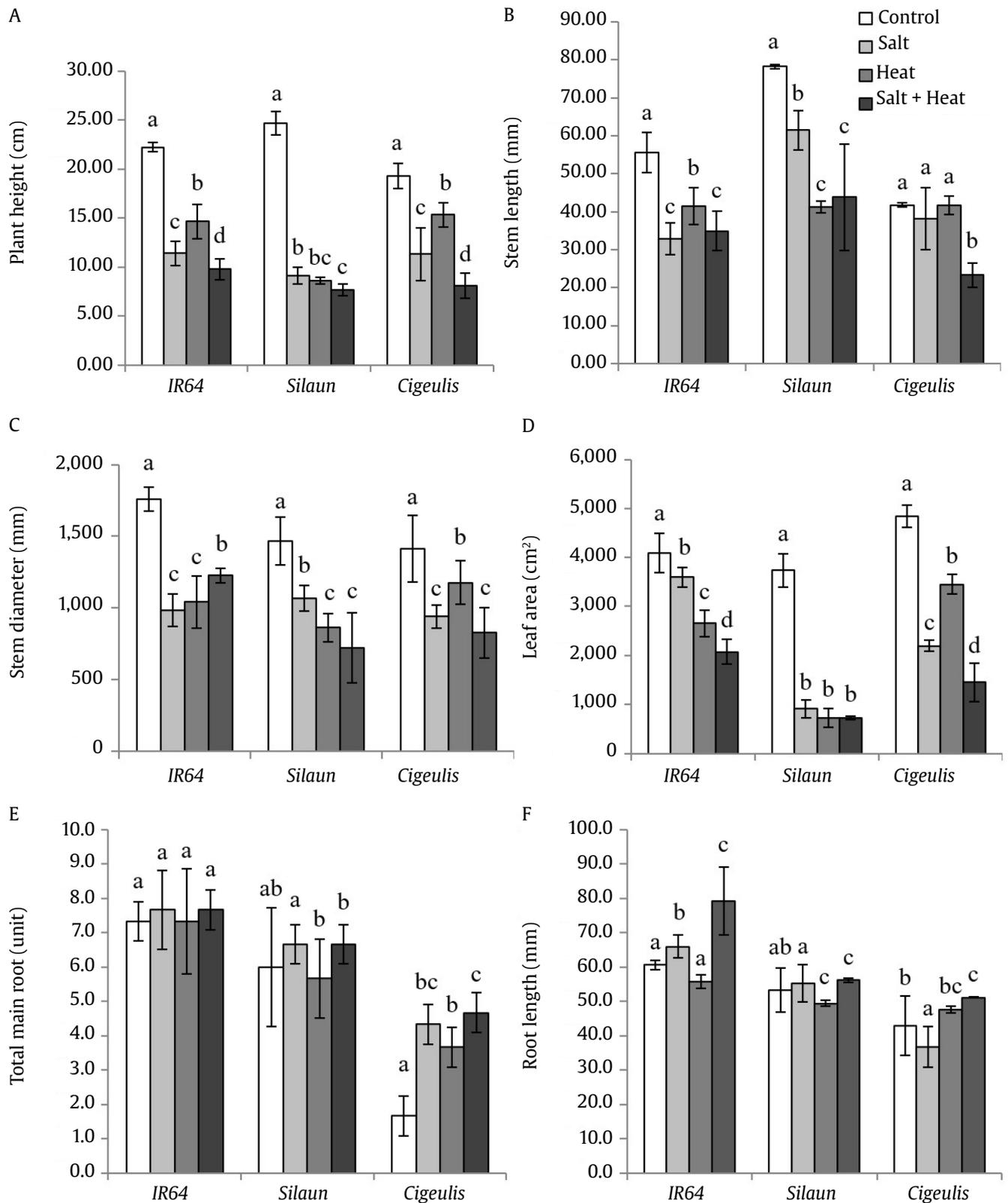


Figure 2. Morphology analysis in *IR64*, *Silaun*, and *Cigeulis* varieties after stress treatments. The parameters: (A) Plant height, (B) Stem length, (C) Stem diameter, (D) Leaf area, (E) Total main root, and (F) Root length. The figure shows that stress treatments (both single and stress combinations) are significantly different at $p < 0.05$ on all parameters

Table 2. The relative values of each variable compared to the control condition under stress combination

Growth variables	Relative decrease or increase (%) ^[1]		
	<i>IR64</i>	<i>Silaun</i>	<i>Cigeulis</i>
Plant height (cm)	56.07↓	68.97↓	57.96↓
Stem length (mm)	37.17↓	43.97↓	44.22↓
Stem diameter (mm)	30.17↓	50.91↓	41.51↓
Leaf area (cm ²)	49.32↓	80.59↓	70.04↓
Total main root (unit)	4.35↑	10.00↑	64.29↑
Root length (mm)	23.53↑	4.91↑	16.10↑
Total fresh weight (gr)	67.47↓	74.88↓	31.23↓
Total dry weight (gr)	41.11↓	45.66↓	15.14↓
Chlorophyll A (mg/g FW)	2.68↓	1.81↑	1.86↑
Chlorophyll B (mg/g FW)	1.18↓	8.07↓	14.26↓
Total chlorophyll (mg/g FW)	1.61↓	5.15↓	8.80↓
Relative water content (%)	3.45↓	18.80↓	9.40↓
H ₂ O ₂ (μM/g FW)	0.68↑	0.81↑	0.58↑

^[1]Relative decrease or increase compared to no stress condition; ↓ indicates relative decrease; ↑ indicates relative increase

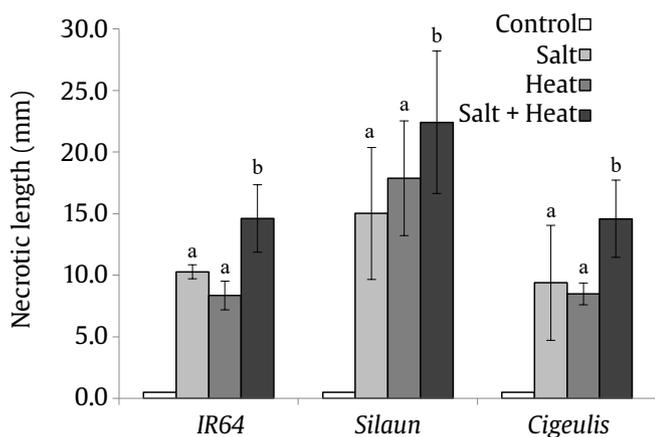


Figure 3. The necrotic length in *IR64*, *Silaun*, and *Cigeulis* varieties after 5 days of treatment. The figure shows that stress treatments (both single and stress combinations) are significantly different at $p < 0.05$

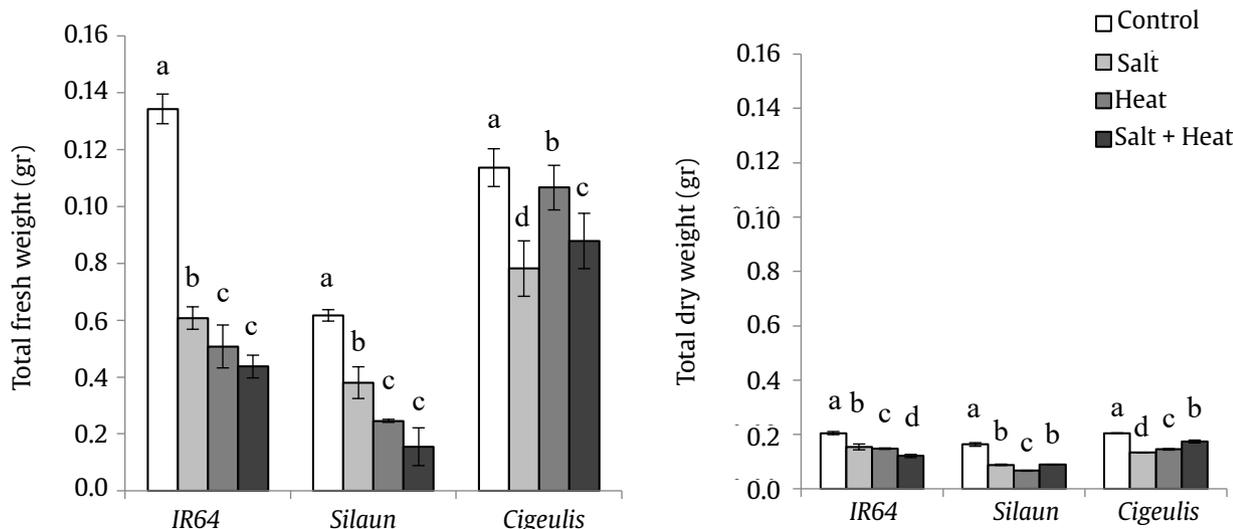


Figure 4. Plant biomass for total fresh weight (left) and total dry weight (right); after stress treatment in plants for 5 days. The figure shows that stress treatments (both single and stress combinations) are significantly different at $p < 0.05$ on all parameters

the morphological parameters (a whole rice plant). The combination of salt and heat stresses in rice caused a significant effect on the total chlorophyll and RWC. The reduction of total chlorophyll due to stress exposure in *IR64*, *Silaun*, and *Cigeulis* varieties was 1.61%, 5.15%, and 8.80%, respectively (Figure 5). Thus, the *Cigeulis* variety obtained the highest total chlorophyll reduction, while *IR64* had the lowest total chlorophyll reduction compared to the two other varieties.

The relative water (RWC) content in *IR64*, *Silaun*, and *Cigeulis* with the combined treatment of salinity (150 mM NaCl) and heat (40°C) stress were reduced at 3.45%, 18.80%, and 9.40%, respectively (Figure 5). Thus, the *IR64* variety obtained the lowest reduction in RWC, while the *Silaun* variety had the lowest reduction in RWC compared to the other two varieties.

The ROS levels in rice plants with stress treatments showed a significant difference compared to controls. The increased ROS levels in *IR64*, *Silaun*, and *Cigeulis* with stress combination treatment were 0.67%, 0.80%, and 0.58%, respectively (Figure 6). The increased ROS production is one of the several plant responses to the abiotic stress, whereas the resistant varieties can commonly produce ROS in small amounts. The *Silaun* variety presents the highest ROS level increase, while the *Cigeulis* variety presents the lowest ROS level increase compared to the other two varieties with the same treatment. Figure 6. indicates that the stress treatments had no significant effect on MDA levels in plants.

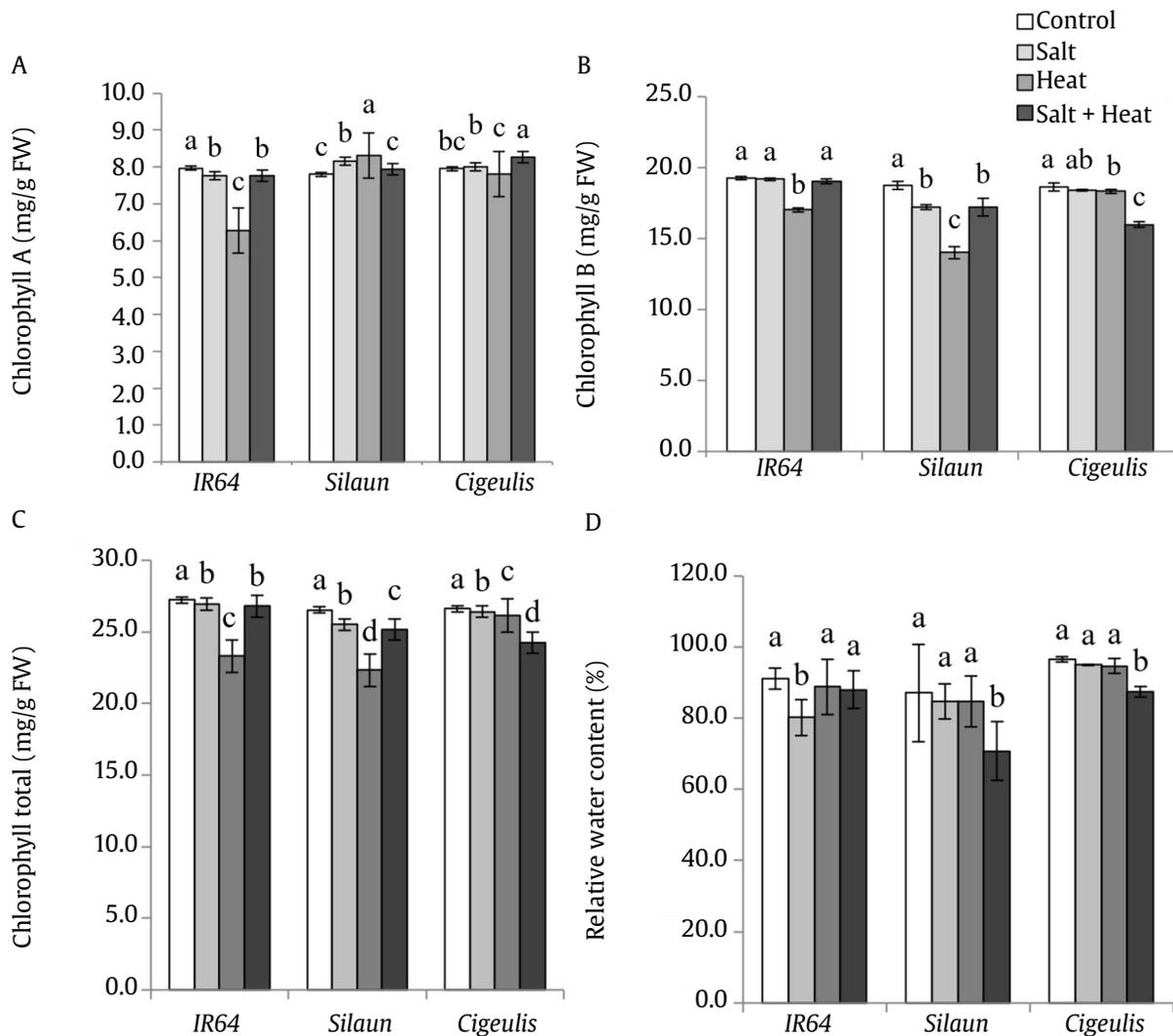


Figure 5. Plant physiology analysis after stress treatments. (A) Chlorophyll A, (B) Chlorophyll B, (C) Total chlorophyll, and (D) Relative water content. The figure shows that stress treatments (both single and stress combinations) are significantly different at $p < 0.05$ on all parameters

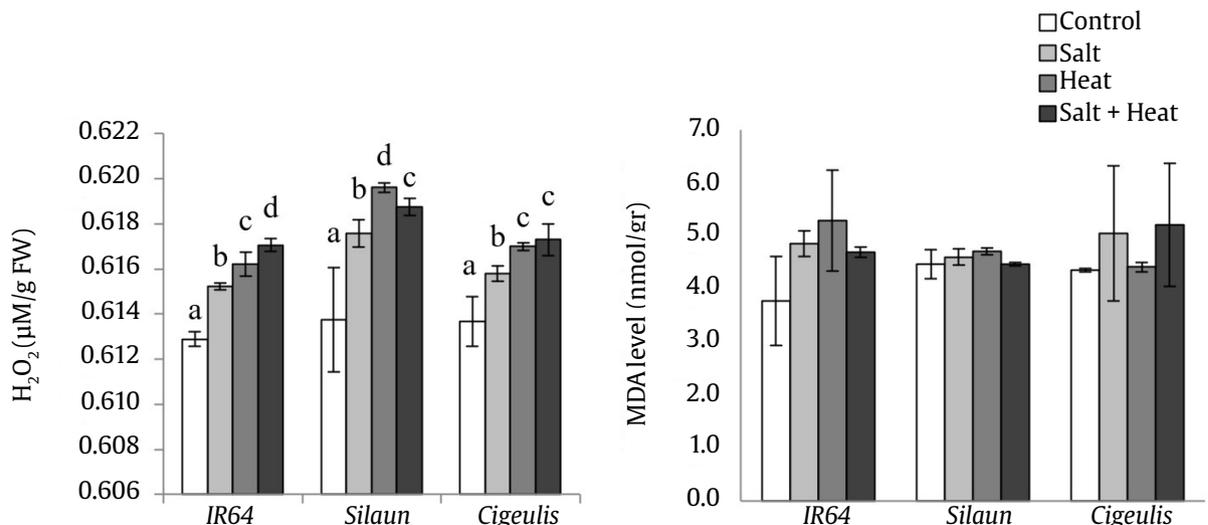


Figure 6. The Quantification of H_2O_2 (left) and malondialdehyde (MDA) (right) in rice plants after stress treatment for 4 hours. The figure shows that stress treatments (both single and stress combinations) are significantly different at $p < 0.05$ on H_2O_2 accumulation. In contrast, stress treatments in local rice did not affect the MDA parameter

3.3. Gene Expression of Antioxidants and Other Metabolites

The *OsActin* is a housekeeping gene that is an internal control in the gene expression analysis that does not affect plant response to stress conditions.

The expression of the *OsNOMT* gene was confirmed in the *Cigeulis* variety after the heat and salt exposure combination treatment. However, the expression of the *OsNOMT* gene was absent in the other two varieties (Figure 7).

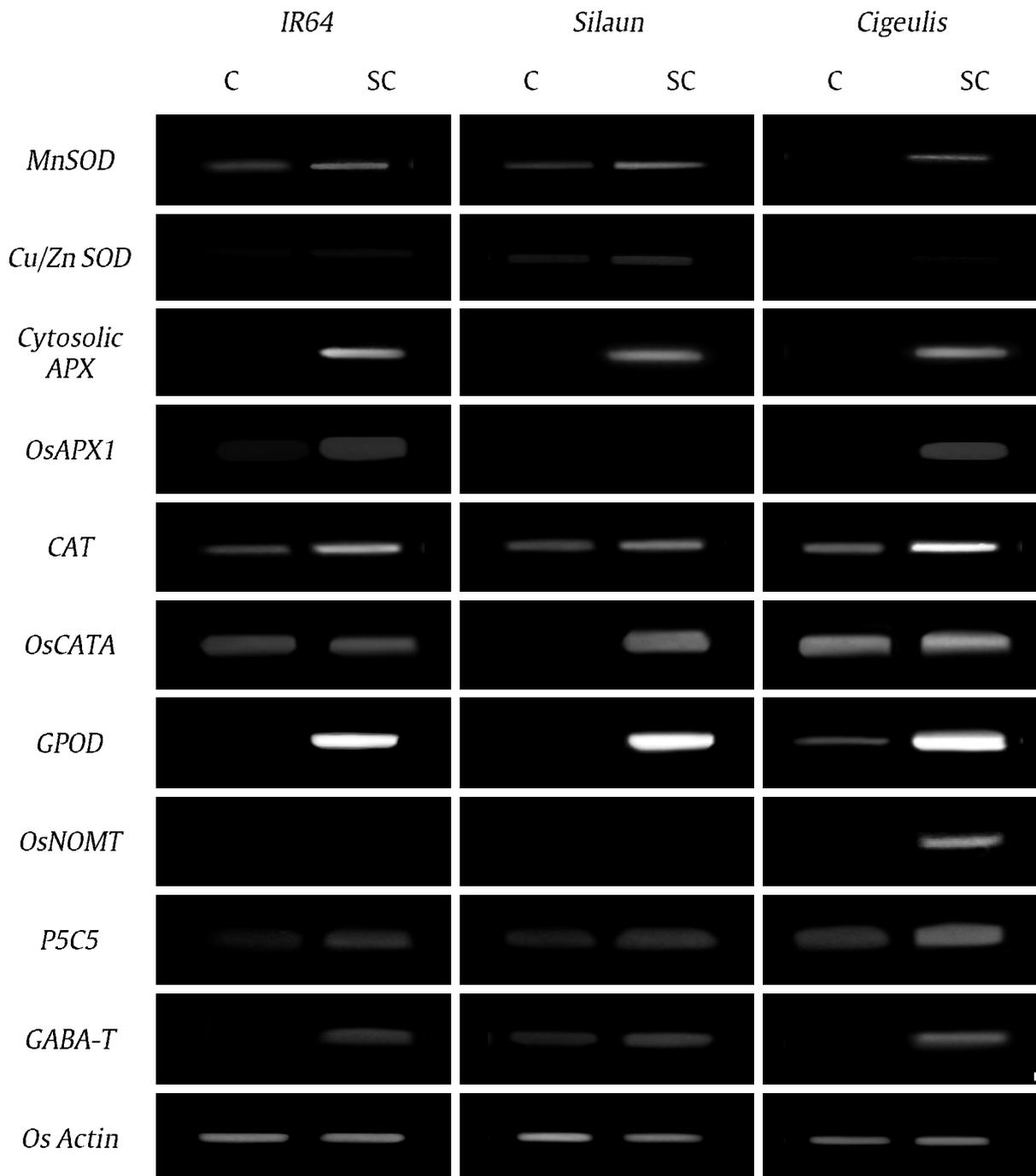


Figure 7. RT-PCR analysis to validate the gene expression in plants after treatments (stored fresh rice leaves at -20°C , after 4 hours of treatments). Seven antioxidant genes were selected and analyzed in all rice varieties (*Mn-SOD*, *Cu/Zn SOD*, *Cytosolic APX*, *OsAPX1*, *CAT*, *OsCATA*, and *GPOD*). Other genes (*OsNOMT*, *P5CS*, and *GABA-T*) are expressed as secondary metabolite products in rice. C: control; : SC: stress combination

The expression of other resistance genes was also confirmed in the *Cigeulis* variety after stress exposure combination treatment. Yet, only one resistance gene remains absent, namely *Cu/ZnSOD*. In the *IR64* variety, all resistance genes, except *OsNOMT*, were also confirmed to appear. The expression of resistance genes for the *Cigeulis* variety was also active after exposed to stress combination, except the *OsNOMT* and *OsAPX1* genes.

These results illustrate that *Cigeulis* has adaptive regulation during stress conditions because this variety can activate the antioxidant and secondary metabolite genes significantly more than other varieties. In gene expression analysis, we did not use statistical analysis, but we observed the thickness of each PCR band product. The statistical analysis was only applied to morphology and physiological parameters.

4. Discussion

The stress combination of salt and heat to plants significantly reduces plant growth. The implications of plant height, stem length and diameter, and leaf area reduction are stunted growth, reduced photosynthesis rate, and nutritional disorder (Khan *et al.* 2021b). Decreased leaf area may become a reflection of a reduction in chlorophyll contents per unit, which can impact the photosynthesis rate during stress conditions (Batoool *et al.* 2020).

After stress treatment, the *Cigeulis* and *Silaun* varieties obtained an increased total main roots and root length (Figure 2). Interestingly, some plants increased total and root length, more pronounced under single stress than under stress combination. This might occur because heat stress is antagonistic to salinity stress (Rivero *et al.* 2014). Bermudez *et al.* (2022) states that each plant, such as tomato (Rivero *et al.* 2014), jatropha (Silva *et al.* 2013), cherry tomatoes (Liu *et al.* 2014), and barley (Faralli *et al.* 2015) has several complex mechanisms to respond, whether to individual or stress combination (Bermúdez *et al.* 2022). A stress combination of salinity and heat can cause increased Na^+ and Cl^- ions and decreased cell absorption of Ca^{2+} around plant roots, a decreased root growth and an increased root/stem ratio, elevating the soil's water and nutrient absorption (Bermúdez *et al.* 2022). Salinity stress causes plants to experience osmotic, ionic stress, and stomatal closure due to the increased H_2O_2 around the guard

cells (Castillo *et al.* 2007; Huang *et al.* 2009; Nawaz *et al.* 2010). Osmotic stress can interfere with water and nutrient absorption by roots (Nawaz *et al.* 2010; Safdar *et al.* 2019), whereas ionic stress occurs due to the high concentration of toxic ions such as Na^+ , which results in cell damage (Nemati *et al.* 2011). In this study, the H_2O_2 accumulation (part of ROS) was observed, but we did not analyze the stomatal closure directly. The heat stress causes changes in plasma membrane fluidity, thus affecting the ion transport around the cell.

Figure 3 shows the necrotic length in the three varieties. The *Silaun* variety had the highest severity level compared to the two other varieties, while the *Cigeulis* variety was more tolerant with the lowest necrotic length after stress treatments. Necrosis indicates a decreased chlorophyll content and cell death in the leaf area (Jan *et al.* 2021). The combination of salinity and heat stresses also causes plant leaves to experience necrosis, characterized by a dry and brownish-yellow leaf surface (Hameed *et al.* 2011; Acosta-Motos *et al.* 2017).

Figure 4 shows that the fresh weight biomass of the three local rice varieties decreased significantly between the control treatments with stress combination. Interestingly, the dry weight reduction with stress combination was smaller than with the single stress in the *Silaun* and *Cigeulis* varieties. This condition occurs because of the different mechanisms of each rice variety in responding to stress, including the signaling pathways for stress combination (Nahar *et al.* 2022). The antagonist mechanism between salt and heat stress can occur because high temperatures induce salinity tolerance by inhibiting Na^+ ions uptake in shoot areas but directing them to the root organ (Rivero *et al.* 2014).

The combination of salinity and heat stresses in rice plants also affects the decreased plant chlorophyll content. Figure 5 shows a significant decrease in chlorophyll b and total chlorophyll content after treatment exposure. The *Cigeulis* variety had the highest total chlorophyll degradation, while the *IR64* variety was tolerant with the lowest degradation value. However, the chlorophyll content in the *Silaun* and *Cigeulis* varieties was increased after the plants were exposed to single and combined stress. The increasing chlorophyll content indicates that the cultivars (*Silaun* and *Cigeulis*) can tolerate abiotic stress conditions with photosynthetic and growth rate improvement (Godoy *et al.* 2021). After salinity

and heat exposure, The decreased chlorophyll content occurred due to oxidative stress caused by the high ROS content in cells (Nahar *et al.* 2022). The decreased chlorophyll content is also one of the plant's defense responses to abiotic stresses, such as salinity and heat, by reducing light absorption so photosynthesis can continue slowly (Taïbi *et al.* 2016).

Relative water content (RWC) was carried out to determine the physiological response of plants in stress conditions. Figure 5 shows a decreased RWC in the three varieties after stress exposure. The highest decrease was found in the *Silaun* (susceptible) varieties, followed by *Cigeulis* and *IR64* (tolerant) varieties after stress combination treatments. The percentage of RWC in leaves indicates the capability of plant cells to store water when facing stress. A higher percentage of RWC describes the plant tolerating certain stresses (El-Bassiouny and Bekheta 2005; Rafique *et al.* 2019). A decreased ability of cells to store water affects plant cell growth and inhibits the nutrient transformation process around the cell wall (Nahar *et al.* 2022).

The quantification of ROS levels was carried out to determine the osmotic stress level experienced by plants. Figure 6 describes that saline, heat, or a combination of salt and heat stress increases plant ROS production. The *Silaun* variety (susceptible) obtained the highest ROS production compared to the *IR64* and *Cigeulis* (tolerant) varieties. Each variety exhibits unique responses when dealing with stress, especially the *Silaun* variety, which has the highest ROS production in plants that experience heat stress. This can be possible if the *Silaun* variety is less resistant to heat stress only but will produce high levels of antioxidants to break down ROS when subjected to stress combination treatments. *Salinity* and heat stress cause plants to suffer oxidative stress and increase cell ROS levels (Moradi and Ismail 2007; Li *et al.* 2007; Hoang *et al.* 2019). ROS can include singlet oxygen (1O_2), superoxide anion ($O_2^{\cdot-}$), and hydrogen peroxide (H_2O_2). ROS are generally produced by plants when experiencing stress as a defense response. If production is too high, ROS can cause damage to cell membranes, lipids, and nucleic acids, leading to cell death (Waszczak *et al.* 2018; Khan *et al.* 2021a).

Expression of the antioxidant genes and secondary metabolites was observed by RT-PCR method and electrophoresis visualization. Several antioxidant genes analyzed in this study could

trigger the formation of antioxidant enzymes, namely superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPOD). Due to stress conditions, these enzymes play a role in the ROS scavenging process (Kibria *et al.* 2017; Park *et al.* 2022).

SOD is the first cell defense in controlling the amount of ROS. SOD enzymes are divided into three types based on their cofactors: *FeSOD*, *Cu/Zn SOD*, and *MnSOD*. The *FeSOD* and *Cu/ZnSOD* are mostly found in the chloroplast and cytoplasmic areas, while *MnSOD* is generally located in mitochondria. SOD will change O_2 to H_2O_2 (Tounsi *et al.* 2019). In this study, there were two types of SOD gene expression: *Cu/Zn SOD* and *MnSOD*. The three local rice varieties produced SOD after stress combination exposure treatments. The *IR64* variety showed the thickest *MnSOD* band, while the *Silaun* variety showed the thickest band in the *Cu/Zn SOD* gene after stress combination exposure. Therefore, the *IR64* and *Silaun* varieties produce a higher SOD enzyme accumulation than the *Cigeulis* variety after the salt and heat stress combination.

ROS in H_2O_2 will be broken down into $H_2O + O_2$ with the help of antioxidant enzymes, such as CAT, APX, and GPOD. CAT can convert ± 6 million H_2O_2 molecules into H_2O and O_2 every minute (Filiz *et al.* 2018; Khan *et al.* 2021a). In this study, two genes formed the CAT enzyme: *CAT* and *OsCATA*. Figure 7 presents that the expression of the *CAT* and *OsCATA* genes was confirmed active in the three varieties after stress combination exposure. The *CAT* band thickness was highly visible in the *Cigeulis* variety, while the *Silaun* variety had the thickest *OsCATA* gene expression.

APX is almost similar to CAT, which can break down the H_2O_2 molecules into H_2O and O_2 , but APX has a greater binding capacity for H_2O_2 than CAT (Filiz *et al.* 2018; Khan *et al.* 2021a). In this study, two gene expressions regulated the APX formation: *Cytosolic APX* and *OsAPX1*. Based on Figure 7, the expression of the APX enzyme regulatory gene was confirmed active in the three local rice varieties after stress combination exposure. The *IR64* variety showed the thickest *Cytosolic APX* and *OsAPX1* gene bands compared to the other two varieties after stress combination exposure.

The GPOD enzyme is an antioxidant enzyme that reduces H_2O_2 molecules in cells using glutathione (GSH), which can minimize cell damage (Brigelius-

Flohe and Flohe 2020). This enzyme is abundant when plants are exposed to abiotic stresses, such as salt and heat stress (Filiz *et al.* 2018). This study analyzed GPOD gene expression in three local rice varieties after being given stress combination treatments. Figure 7 shows that three local rice varieties were confirmed to produce GPOD enzymes. The *Cigeulis* variety showed the thickest gene expression band compared to the other two local rice varieties. Based on these results, each local rice variety has a different mechanism in responding to stress, including stress combination.

Figure 7 depicts the expression of the *OsNOMT* gene in the *Cigeulis* variety, following the treatment with combining stressors. This condition is possible because the *Cigeulis* variety is a tolerant rice cultivar to the *Pyricularia oryzae* pathogen (Santoso *et al.* 2022). The *P. oryzae* infection in rice plants has been confirmed to trigger the production of sakuranetin (substrate of naringenin biosynthesis with the help of the NOMT enzyme). The *OsNOMT* gene expression is also affected by the activation of the *OsNOMT* promoter by *OsMYC2*. The *OsMYC2* is a transcription factor of JA hormone signaling. *OsMYC2* will cooperate with *OsMYC2*-like proteins 1 and 2 (*OsMYL1* and *OsMYL2*) to induce the production of sakuranetin in plants as a response to plant defense against stress conditions (Ogawa *et al.* 2017).

The expression of the *P5CS* gene was evident in the *Cigeulis* variety, followed by the *Silaun* and *IR64* varieties. The *P5CS* (Δ^1 -pyrroline-5-carboxylate synthetase) is a gene that regulates the formation of proline in cells. Proline increases nutrient absorption through plasma membrane H^+ -ATPase activity enhancement (Hayat *et al.* 2021), antioxidant activity, and ROS scavenging mechanism support in cells when plants are exposed to abiotic stress. Proline also controls ROS production in mitochondria (El-Moukhtari *et al.* 2020).

In addition to proline, there is also GABA, which acts as an antioxidant acceptor. In this study, *GABA-T* gene expression was analyzed in three local rice varieties after stress combination exposure. In Figure 7, the *Silaun* variety has the thickest PCR bands, followed by the *IR64* and *Cigeulis* varieties. However, the PCR band thickness for the three local rice varieties had no significant difference. GABA is generally produced through the GABA shunt pathway mechanism. This mechanism effectively suppresses ROS formation in plants

and regulates the activity of antioxidant enzymes. *GABA transaminase (GABA-T)* acts as a catalyst for converting GABA to Succinic Semialdehyde (SSA). The *GABA-T* can also indicate GABA production in cells (Li *et al.* 2021).

From this study, we conclude that stress combination treatments can affect rice's morphology and genetic parameters. In the morphological parameters, the stress combination of salt and heat reduced the plant height, stem length, stem diameter, and leaf area. The combination of salt and heat stresses also increased the root length and the total main roots. This stress treatment induced leaf necrosis at all abiotic stress treatments. In this study, the stress combination influenced the physiological parameters, including RWC, chlorophyll reduction, and H_2O_2 accumulation in the rice plants. In genetic parameters, the combination of salt and heat stresses can highly express the antioxidant genes (*Mn-SOD*, *Cu/Zn SOD*, *Cytosolic APX*, *OsAPX1*, *CAT*, *OsCATA*, and *GPOD*) and secondary metabolite genes (*P5CS*, *GABA-T*, and *OsNOMT*) in all varieties. This study also finds the *Cigeulis* variety as the most resistant rice among the other varieties used in this study. This condition describes that *Cigeulis* can activate the *CAT*, *GPOD*, *OsNOMT*, and *P5CS* gene expression (Figure 6). This *Cigeulis* variety is more stable and became the most resistant based on all observed characters than *IR64* and *Silaun* varieties (Table 2).

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