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Impact of salinity stress on rice regeneration and molecular defense: insights from IR64 and Cigeulis varieties

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Abstract

Salinity stress is a major abiotic factor that significantly reduces crop productivity. This study examines the impact of salinity stress on the expression of antioxidant enzymes in two rice varieties, IR64 and Cigeulis. Exposure to 150 mM NaCl induced the highest browning intensity in callus tissues, corresponding to reduction in callus diameter. Morphological changes during regeneration followed distinct developmental stages, including globular, scutellar, coleoptile, and plantlet phases. Salinity stress strongly influenced browning levels, callus size, and embryogenic potential, all showing a positive correlation with increasing NaCl concentrations. At the molecular level, defense-related genes, including *Oryza sativa* catalase A (*OsCATA*), manganese superoxide dismutase (*MnSOD*), cupper/zinc superoxide dismutase (*Cu/ZnSOD*), cytosolic ascorbate peroxidase (*cytosolic APX*), *Oryza sativa* ascorbate peroxidase (*OsAPX1*), and pyrroline-5-carboxylate synthetase (*P5CS*), exhibited enhanced expression, as indicated by intensified DNA band signals. These results suggest that salinity stress activates the rice antioxidant defense system. Notably, IR64 and Cigeulis maintained their regenerative capacity under 150 mM NaCl, forming globular-like structures in the callus, demonstrating a degree of tolerance to salinity stress.

Keywords: antioxidant, callus regeneration, embryogenic, gene expression, salinity stress.

Introduction

Over the past few decades, rice (*Oryza sativa* L.) has remained a fundamental staple crop and a primary nutritional energy source for nearly 3.5 billion people worldwide, particularly in Asia. With the global population projected to reach 9.6 billion by 2050, rice production must significantly increase to meet the escalating food demand. However, salinity stress poses a major abiotic challenge that severely hampers plant growth and productivity. Soil salinization, driven by climate change and rising temperatures, leads to an excessive accumulation of salts in the soil (Sári et al., 2023). This phenomenon disrupts plant physiology through water deficit, cytotoxic effects of Na^+ and Cl^- ion accumulation, and nutrient imbalances (Isayenkov and Maathuis, 2019). In coastal regions, salinity stress is further intensified by seawater intrusion into groundwater reserves (Muhardi et al., 2020), while in arid and semi-arid areas, low rainfall limits salt leaching, resulting in excessive salt accumulation (Karolinoerita

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Abbreviations: CAT - catalase; Cu/ZnSOD - cupper/zinc superoxide dismutase; cytosolic APX - cytosolic ascorbate peroxidase; DMRT - *Duncan*'s Multiple Range Test; EtBr - ethidium bromide; GABA-T - γ-aminobutyric acid transaminase; GPOD - guaiacol peroxidase; GR - glutathione reductase; MDA - malondialdehyde; MnSOD - manganese superoxide dismutase; OsAPX1 - *Oryza sativa* ascorbate peroxidase; OsCATA - *Oryza sativa* catalase A; P5CS - pyrroline-5-carboxylate synthetase; Q-PCR - quantitative polymerase chain reaction; ROS - reactive oxygen species; SOD - superoxide dismutase; SPSS - Statistical Package for Social Sciences; WAT - week after treatment.
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and Yusuf, 2020). Exposure to salinity stress induces the overproduction of reactive oxygen species (ROS), a group of highly reactive free radicals that can damage essential cellular components, including DNA, proteins, lipids, and pigments, ultimately impairing plant function (Ghosh et al., 2021). To mitigate these detrimental effects, plants activate various adaptive responses (Huong et al., 2020), including the upregulation of antioxidant enzyme systems (Jan et al., 2019), which play a crucial role in ROS scavenging and oxidative stress alleviation. These responses involve both well-developed enzymatic and non-enzymatic scavenging pathways or detoxification systems to counter the destructive effects of ROS that include the enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), and so forth (Hasanuzzaman et al., 2011).

During abiotic stresses, SOD catalyzes the dismutation of $O_2^{\cdot-}$ into O_2 and H_2O_2. The CAT converts H_2O_2 to O_2. GR catalyses the conversion of oxidized glutathione (GSSG; dimeric) to reduced glutathione (GSH; monomeric). APX utilizes ascorbate as a specific electron donor to scavenge H_2O_2 to water (Rajput et al., 2021). These enzyme mechanisms enable plants to cope with oxidative stress and maintain the redox balance during unfavorable environmental conditions. In response to stress, plants enhance the activity of their antioxidative capacity by activating the gene expression and post-translational modifications of key enzymes (Rao et al., 2025). Excessive ROS accumulation disrupts ion homeostasis within the cell, leading to damage in primary cellular structures (Chen et al., 2021). Rice is susceptible to salinity stress, which significantly reduces its yield. Salt-sensitive rice varieties tend to accumulate higher concentrations of Na^+ in leaves and shoots compared to salt-tolerant cultivars (Liu et al., 2019). Various factors, including the growth environment and tolerance index influence the plant's response to salinity stress. Traditional approaches for studying plant resistance mechanisms often rely on *in vivo* or direct planting techniques (Challagulla et al., 2015). However, these methods are susceptible to environmental variability, leading to broad and non-specific physiological responses. Moreover, *in vivo* plants comprise differentiated organs and tissues, each contributing distinct mechanisms resulting in complex physiological outcomes. In contrast, *in vitro* culture techniques provide a controlled and reproducible environment for investigating plant stress responses. *In vitro* culture has been widely employed as a powerful technique for enhancing and analyzing plant resistance responses to biotic and abiotic stress. These methods allow plant cells to revert to an undifferentiated state, resembling stem cells, before functional specialization occurs. This unique characteristic makes *in vitro* systems particularly advantageous for dissecting specific stress response pathways with greater precision. Consequently, studies employing *in vitro* rice culture offer valuable insights into the molecular and physiological mechanisms underlying salinity tolerance in different rice varieties.

This study aims to characterize callus regeneration and antioxidant enzyme gene expression in rice under salinity stress using *in vitro* culture. Specifically, we will analyze the cellular responses of two rice varieties: Cigeulis, a locally available salinity-tolerant variety (Bdr et al., 2020), and IR64, a moderately tolerant control variety. Cigeulis was developed by BB Padi (the Indonesian Center for Rice Research) and released in 2002 by Lampung Agricultural Technology Assessment Center (Lesmana et al., 2004). On the other hand, IR64 was developed by the International Rice Research Institute (IRRI) and released in 1986. Both varieties were selected for their agronomic relevance and differing responses to abiotic stress. By leveraging *in vitro* techniques, this research seeks to elucidate the cellular defense mechanisms activated under salt stress conditions, providing deeper insights into the molecular basis of salinity tolerance in rice.

Materials and methods

Callus induction: The study utilized two rice varieties, Cigeulis and IR64, as experimental plant materials. Rice seed embryos were carefully excised and surface sterilized by immersing them in 30% sodium hypochlorite, shaking for 15 min, and rinsing three times with sterile distilled water (Kim et al., 2022). The embryos were then blotted dry using sterile filter paper before inoculation. Sterilized seeds were cultured on a callus induction medium consisting of 4.43 g L^{-1} N6 basal salts, 1 mL L^{-1} 2,4-D, 30 g L^{-1} sucrose, 0.3 g L^{-1} casamino acid, 0.1 g L^{-1} myo-inositol, and 4 g L^{-1} gelrite (Jan et al., 2020). For salinity stress treatments, NaCl was supplemented at concentrations of 0 mM (P0), 100 mM (P1), and 150 mM (P2) in the induction medium (Ubaidillah et al., 2024). The medium components were homogenized using a magnetic stirrer, and the pH was adjusted to 5.8 before autoclaving at 121°C and 17.5 psi for 30 min. Under sterile conditions, approximately 20–25 mL of the medium was dispensed into Petri dishes. Seeds were placed on the induction medium with the embryo side in direct contact with the surface and incubated at 25 ± 2°C in complete darkness (Saharan et al., 2004). Callus formation was evaluated two weeks post-culture by assessing callus induction percentage, callus diameter, and morphological characteristics under a microscope. Morphological observations included color, texture, and degree of browning, which were monitored weekly, while callus diameter was quantified using *ImageJ* software. Parameters related to green spot formation and browning intensity were also measured using *ImageJ* software, based on their respective color intensity values. The most promising calluses from the induction phase were subcultured onto fresh treatment media and incubated for an additional two weeks. Each treatment was conducted with three biological replicates, each containing five calluses. Weekly observations were performed to monitor callus growth dynamics, including changes in size, browning intensity, and overall morphology.

Regeneration of plants under salinity stress: Two-week-old embryogenic calluses derived from the

induction medium were transferred to a regeneration medium consisting of 4.43 g L^{-1} MS basal salts, 1 mg L^{-1} NAA, 2 mg L^{-1} kinetin, 0.3 g L^{-1} casamino acid, 30 g L^{-1} sucrose, 4 g L^{-1} gelrite, and supplemented with NaCl at concentrations of 0 mM (control), 100 mM, and 150 mM. Cultures were maintained under a 16-h photoperiod at a light intensity of 33 µmol(photon) m^{-2} s^{-1} and a controlled temperature of 24°C. Each treatment comprised three biological replicates, with five calluses per replicate. Regenerated plantlets were subsequently transferred to fresh regeneration medium in Petri dishes to promote shoot elongation. Regeneration responses were assessed during the first week, focusing on green spot formation and browning intensity. Additionally, the developmental progression of calluses was monitored by recording the number of structures transitioning into globular, scutellar, and coleoptile phases during the early stages of plant regeneration.

Cytological characterization of callus: Cytological analysis of rice callus was performed for each treatment using 2% acetocarmine and 0.5% Evans blue staining. Calluses collected from the second and fourth weeks of induction, as well as the first week of regeneration, were subjected to staining procedures to assess cellular viability and structural characteristics. For staining, calluses were immersed in 2% acetocarmine for 2 min, followed by three rinses with sterile distilled water to remove excess stain. Subsequently, samples were treated with 0.5% Evans blue for 30 s, then rinsed thoroughly to eliminate residual dye. Stained calluses were examined under a light microscope, and morphological measurements were analyzed using *ImageJ* software.

Gene expression analysis of antioxidant and metabolite-related genes: The study analyzed the expression patterns of antioxidant enzyme genes (*OsCATA*, *CAT*, *MnSOD*, *Cu/ZnSOD*, *cytosolic APX*, *OsAPX1*, and guaiacol peroxidase *GPOD*) and metabolite-related genes associated with GABA metabolism (γ-aminobutyric acid transaminase; *GABA-T*) and proline biosynthesis (*P5CS*). Callus samples were collected at the second and fourth weeks of induction and the first week of regeneration. Based on Mufadilah et al. (2024), gene expression analysis was conducted in three main steps, with the specific primers provided in Table 1 Suppl. (Kim et al., 2007; 2018). First, total RNA was extracted using the *Ribospin™ Plant Kit* (*GeneAll*, Seoul, South Korea) following the manufacturer's protocol. Next, complementary DNA (cDNA) was synthesized using the *ReverTra Ace® qPCR Master Mix* (*Toyobo*, Osaka, Japan). Finally, quantitative polymerase chain reaction (Q-PCR) was performed using *GoTaq® Green Master Mix* (*Promega*, Madison, USA) in a 15 µL reaction volume. The amplified Q-PCR products were separated on a 2% agarose gel, stained with ethidium bromide (EtBr), and visualized under a UV transilluminator. Gene expression levels were assessed based on the intensity and thickness of the resulting fluorescent DNA bands, which were documented and relative gene expression levels were quantified by analyzing the fluorescence intensity of PCR bands using *ImageJ* software. Band intensity values were normalized to a housekeeping gene (*Actin*) and expressed as fold change relative to the control treatment.

Statistical analysis: All experiments were conducted in a completely randomized design with $n = 3$ biological replicates per treatment. Data are presented as mean ± standard deviation (SD). For each variety, the effects of salt stress treatments were analyzed separately using one-way analysis of variance (*ANOVA*). When significant differences were detected ($P \leq 0.05$), mean separation was performed using *Duncan*'s Multiple Range Test (DMRT). All statistical analyses were performed using *IBM SPSS* statistics version *26.0* (*IBM Corp*., Armonk, NY, USA). In cases where only two treatments were compared (*e.g.*, Greenspot %), an independent *t*-test was applied, and no post-hoc letters were assigned.

Results

Cell dedifferentiation in IR64 and Cigeulis varieties: Cell dedifferentiation in the IR64 and Cigeulis rice varieties began during the induction stage, where seed explants transitioned into callus cells following stimulation. After two weeks, distinct callus formation was observed, with the embryogenic callus exhibiting a compact structure and a white-yellowish color, suggesting embryogenic potential (Fig. 1 Suppl. and Table 1).

Morphological characteristics of callus after treatment: Significant differences in morphological characteristics were observed between the control treatment (P0) and the 150 mM NaCl treatment (P2) in both the IR64 and Cigeulis rice varieties (Table 2 and Fig. 1). However, browning of the callus, a sign of stress, became apparent as the tissue turned dark brown, particularly under the 150 mM (P2) NaCl treatment. This browning was accompanied by a noticeable reduction in callus diameter and shrinkage, with the most pronounced effects observed at two weeks after treatment (2 WAT). In contrast, calluses from both the IR64 and Cigeulis varieties under the control treatment displayed no significant browning or shrinkage.

The percentage of browning in the control treatment was 5.52% for IR64 and 5.25% for Cigeulis, with no significant statistical variation. However, under 100 mM NaCl treatment (P1), browning increased to 17.0% for IR64 and 22.45% for Cigeulis. The highest browning percentages were recorded at 150 mM NaCl, where they reached 32.75% for IR64 and 37.72% for Cigeulis, with Cigeulis showing a notably higher browning rate. Callus

Table 1. Morphological characteristics of IR64 and Cigeulis callus. Diameter values are the means ± SD ($n = 3$).

Variety	Color	Induction percentage	Diameter (mm)	Texture
IR64	White yellowish	97.22%	5.24 ± 1.80	Compact
Cigeulis	White yellowish	93.08%	3.87 ± 0.45	Compact

Table 2. Percentage of callus browning two weeks after treatment. Values are the means ± SD (n = 3). *Different letters* indicate significant differences between the control and treatments ($P \leq 0.05$) as determined by *Duncan*'s Multiple Range Test (DMRT).

Variety	Treatment	Browning level (%)
IR64	Control (P0)	5.52 ± 2.75[a]
IR64	100 mM NaCl (P1)	17.00 ± 12.3[ab]
IR64	150 mM NaCl (P2)	32.75 ± 9.38[b]
Cigeulis	Control (P0)	5.25 ± 4.52[a]
Cigeulis	100 mM NaCl (P1)	22.45 ± 13.79[ab]
Cigeulis	150 mM NaCl (P2)	37.72 ± 8.13[b]

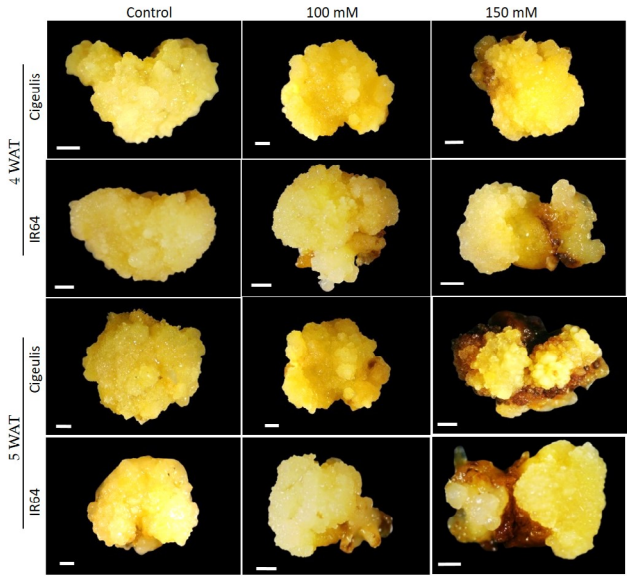

Fig. 1. Representative callus morphology at 4 and 5 weeks after treatment with different concentrations of NaCl (scale bars = 1 mm). Control - 0 mM NaCl, 100 mM - 100 mM NaCl, 150 mM - 150 mM NaCl.

diameter also showed a clear decrease with increasing salinity concentration and treatment duration (Table 3). Under the 150 mM NaCl treatment (P2), callus diameter decreased by approximately 29.94% in Cigeulis and 27.63% in IR64 compared to the control. At 100 mM NaCl (P1), the reductions were around 14.66% and 12.54% for Cigeulis and IR64, respectively. Among all treatments, the samples subjected to 150 mM NaCl (P2) exhibited the smallest diameters. In contrast, no significant difference in callus size was observed between the 100 mM NaCl treatment (P1) and the control treatment (P0). Furthermore, a visual trend was observed between callus size and the percentage of browning, suggesting that increased browning may be linked to a reduction in callus size.

Characteristics of callus regeneration: Calluses were cultured for one month on MS medium during the regeneration phase, leading to the formation of roots and leaves in both IR64 and Cigeulis varieties. The regeneration responses to salinity treatments were

Table 3. Callus diameter two weeks after treatment. Values are the means ± SD (n = 3). *Different letters* indicate significant differences between the control and treatments ($P \leq 0.05$) as determined by *Duncan*'s Multiple Range Test (DMRT).

Variety	Treatment	Diameter (mm)
IR64	Control (P0)	8.69 ± 0.67[a]
IR64	100 mM NaCl (P1)	7.60 ± 1.01[ab]
IR64	150 mM NaCl (P2)	6.29 ± 0.79[b]
Cigeulis	Control (P0)	8.15 ± 0.88[a]
Cigeulis	100 mM NaCl (P1)	6.96 ± 0.79[ab]
Cigeulis	150 mM NaCl (P2)	5.71 ± 0.45[b]

compared to the control to assess plant development under both optimal and stress conditions. The initial phase of regeneration was marked by the emergence of green spots (Fig. 2*A*), a key indicator for evaluating regeneration potential. The percentage of green spots exhibited an inverse correlation with salinity concentration. In IR64, the green spot percentage was 6.78% under the control treatment and decreased to 6% under 150 mM NaCl (P2). In Cigeulis, the corresponding values were 2.41% and 1.81%, respectively (Table 4).

Three distinct regeneration phases, such as globular, scutellar, and coleoptilar, were observed in both varieties (Fig. 2*B*). These phases began within the first week after subculturing onto the MS regeneration medium. Salinity stress significantly affected the growth characteristics of both varieties, with more stunted development observed.

Characteristics of embryogenic callus: Embryogenic cells stained red with acetocarmine, while non-embryogenic cells turned blue when exposed to Evans blue. Cigeulis and IR64 callus samples in the control treatment demonstrated a strong response to acetocarmine, with almost the entire callus surface staining red, indicating their embryogenic potential. The percentage of embryogenic callus decreased with increasing salinity concentration (Fig. 3). Under the control treatment (P0), IR64 exhibited an embryogenic percentage of 89%, while Cigeulis showed 80%. The lowest embryogenic percentages were recorded under 150 mM NaCl (P2), 57% for IR64 and 42% for Cigeulis. Embryogenic callus with globular structures, representing a more advanced stage of development, formed during *in vitro* culture, suggesting the callus's ability to withstand salinity stress under these conditions.

Expression of antioxidant and metabolite-related genes: The analysis focused on antioxidant genes and primary metabolite-related genes, including *GABA-T* and *P5CS*. The results indicated that the DNA band intensity increased with higher NaCl concentrations. Fig. 4*A* illustrates the morphological effects of NaCl on regenerated plants, while Fig. 4*B* presents the gene expression levels in callus under different salt stress conditions. Almost all the genes (*OsCATA*, *CAT*, *MnSOD*, *Cu/Zn SOD*, *cytosolic APX*, *OsAPX1*, *GABA-T*, and *P5CS*) were significantly upregulated at 150 mM NaCl in both IR64 and Cigeulis

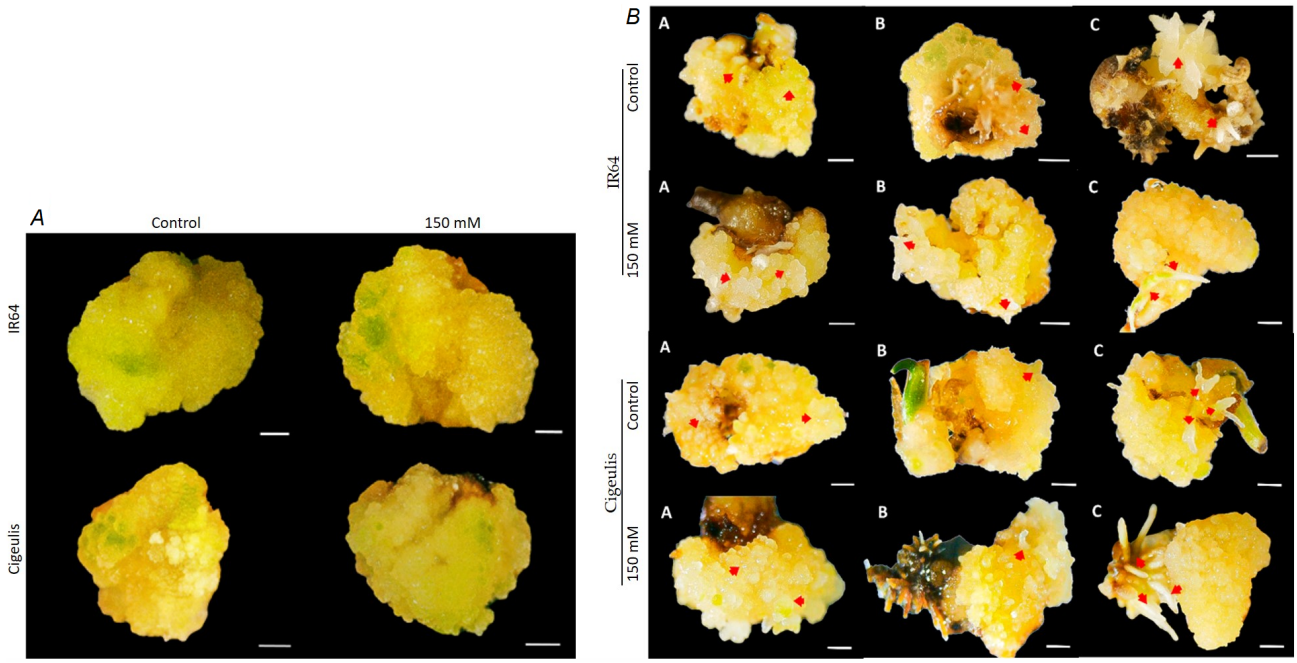

Fig. 2. (*A*) Green spots on rice IR64 and Cigeulis callus appeared one week after 150 mM NaCl treatment on MS regeneration medium (scale bars = 1 mm). (*B*) Stages of rice IR64 and Cigeulis callus regeneration observed one week after 150 mM NaCl treatment, including (a) globular, (b) scutellar, and (c) coleoptilar stages (scale bars = 1 mm). *The red arrows* point to embryogenic nodular structures on the callus. These nodular regions are characterized by compact and meristematic cells with high regeneration.

Table 4. Greenspot percentage in callus after one week of NaCl treatment on MS regeneration medium. Values are the means ± SD (n = 3).

Variety	Treatment	Greenspot (%)
IR64	Control (P0)	6.78 ± 15.2
IR64	150 mM NaCl (P2)	6.00 ± 10.6
Cigeulis	Control (P0)	2.41 ± 8.0
Cigeulis	150 mM NaCl (P1)	1.81 ± 6.0

rice callus, with the exception of *GPOD* expression in Cigeulis, which decreased compared to the control callus. Notably, the expression levels of *OsCATA*, *CAT*, *OsAPX1*, and *GPOD* genes at 150 mM NaCl were lower in Cigeulis than in IR64. The effect of salt stress on callus was observed as a reduction in survival percentage as the NaCl concentration increased (Taratima et al., 2022). In contrast, low concentrations of NaCl stimulate the cells to form tolerance mechanisms in order to survive (Yunita et al., 2014). These findings suggest that the upregulation of these genes in response to salt stress plays a crucial role in mitigating stress during callus regeneration., with each treatment conducted with three biological replicates, each consisting of five calluses.

Discussion

Callus induction is a crucial process that allows plant cells to dedifferentiate and return to a meristematic state, promoting the formation of new tissues (Lailani and Kuswandi, 2023). Both the IR64 and Cigeulis rice varieties showed significant callus induction percentages throughout this phase, indicating superior dedifferentiation ability. Both callus induction exhibited a significant decrease in callus growth and contents of K^+ and Ca^{2+} (Alhasnawi et al., 2017). In addition, significant accumulation of Na^+ and K^+/Na^+ was observed, as well as an enhanced enzymatic antioxidant system and elevated proline activities under NaCl conditions, particularly in the Cigeulis variety. The stability of rice cell membranes under salinity stress is influenced by osmotic adjustment mechanisms, with the increase in Na^+ concentration in the cells, the cellular water potential decreases and affects membrane stability (Tufail et al., 2018). In comparison to Cigeulis, IR64 exhibited greater callus diameters and induction rates. The callus displayed characteristics related to embryogenic callus structures, including compactness, a smooth surface, and a white-yellowish color (Murugesan et al., 2022). Additionally, the callus's compact texture, which reflects closely spaced cells, facilitates effective tissue formation (Warchoł et al., 2015).

The response of rice callus to salinity stress varies depending on the variety and the level of NaCl concentration. Browning in the callus is a key indicator of its response to salinity stress. According to Atabaki et al. (2018), callus growth decreases with increasing NaCl concentration. Salt-sensitive callus is more severely affected, turning brown and watery, often followed by necrotic wrinkling in the darkened areas. In contrast, salt-tolerant callus exhibits less pronounced browning and maintains better cell proliferation potential. Table 2 highlights a significant correlation between increasing

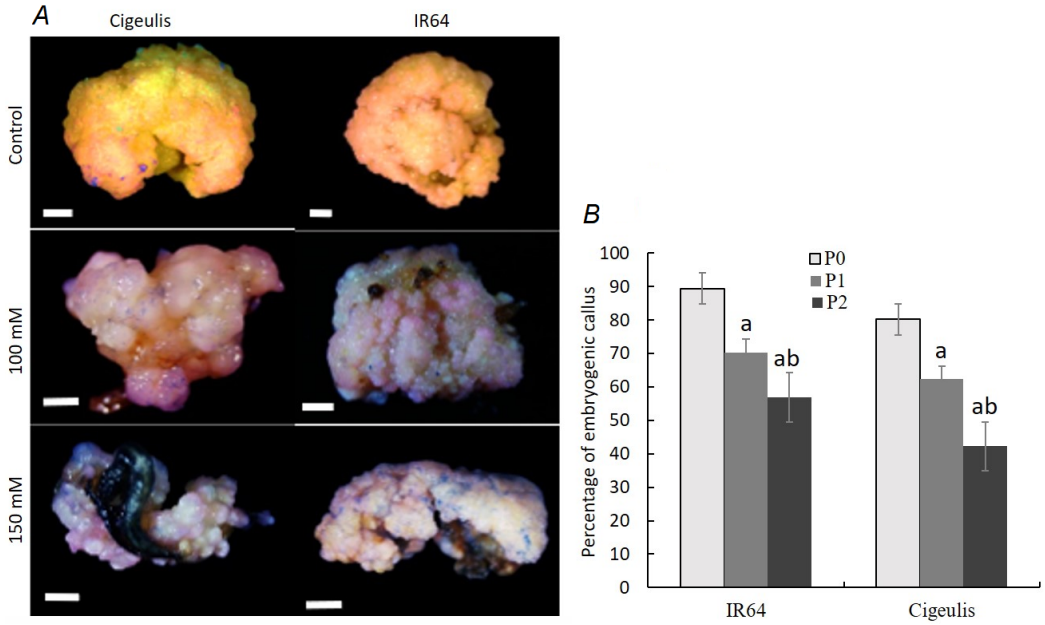

Fig. 3. (*A*) Embryogenic characteristics of rice IR64 and Cigeulis callus one week after treatment (scale bars = 1 mm). (*B*) Statistical analysis was perfomed separately for each variety. Error bars represent standard deviation, while *different letters* indicate significant differences between the control and treatments ($P \leq 0.05$) as determined by *Duncan*'s Multiple Range Test (DMRT).

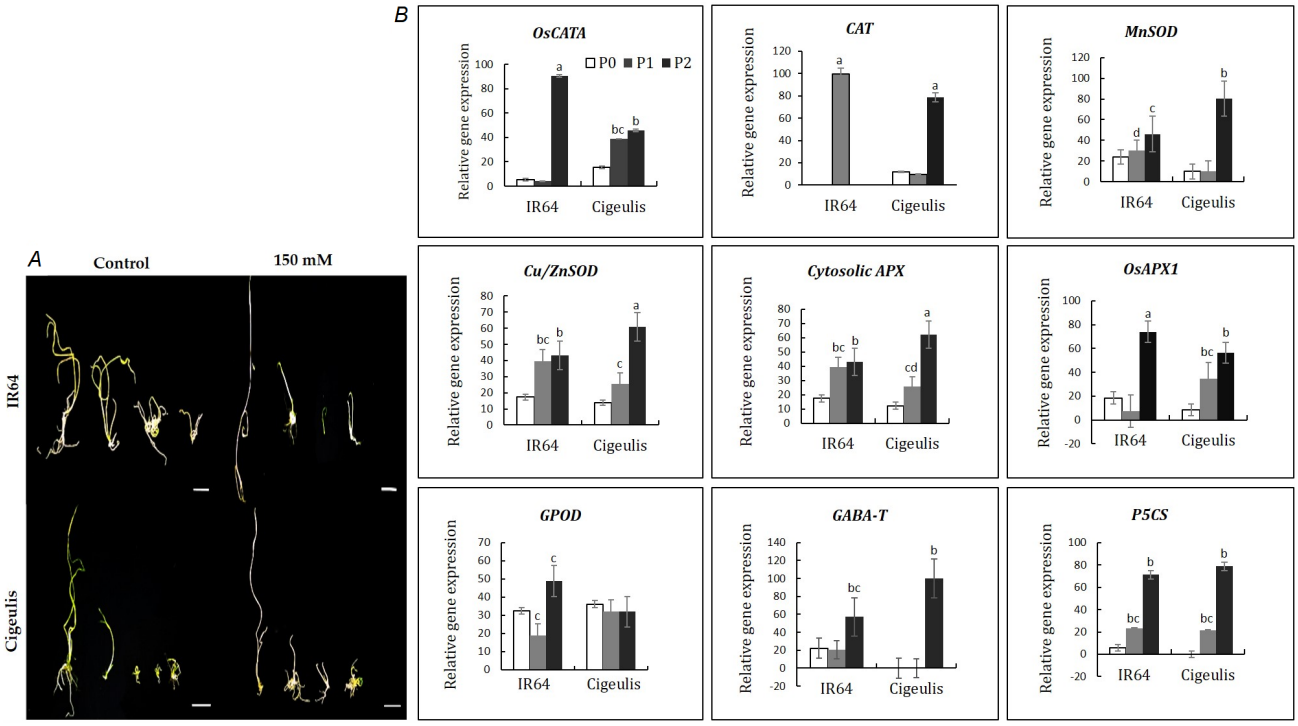

Fig. 4. (*A*) Morphological characteristics of regenerated IR64 and Cigeulis callus after after one month of culture, following two weeks of 150 mM NaCl treatment on MS regeneration medium (scale bars = 1 mm). The variation between the plantlets in the same treatment depicts individual differences in NaCl treatment and regenerative capacity among the callus-derived plantlets. (*B*) Expression levels of antioxidant and metabolite-related genes in the callus of IR64 and Cigeulis. P0 – 0 mM NaCl, P1 – 100 mM NaCl, P2 – 150 mM NaCl. Error bars represent standard deviation, while *different letters* indicate significant differences between the control and treatments ($P \leq 0.05$) as determined by *Duncan*'s Multiple Range Test (DMRT).

NaCl concentrations and a higher degree of browning. Only a small percentage of rice callus cells remain stable and healthy under prolonged salt stress, leading to visible color changes and restricted development (Fig. 1). The color transition from white-yellowish to brown and then to dark brown reflects a stress response in the callus.

This response is linked to the activation of specific enzymes that stimulate the production of phenolic compounds, serving as a self-defense mechanism (Rismayanti and Nafi'ah, 2021). To maintain the cellular osmotic balance under saline conditions, plants experienced a significant increase in proline, malondialdehyde (MDA), and glutathione reductase (GR) concentrations as compared with the unstressed conditions (Sheteiwy et al., 2019). Additionally, the concentration and duration of salt stress exposure result in a significant reduction in callus diameter, as shown in Table 3. These findings indicate that salinity stress imposes both morphological and biochemical changes on rice callus, with its impact varying based on the callus's salt tolerance capacity. The observed browning and reduced survival of callus under salinity stress may be related to physiological mechanisms reported in whole rice plants. Drought stress adversely affects the overall morphology of rice plants, disrupting plant growth, plant biomass and yield, roots, and grain development. Morphologically, water stress exhibits decreased germination, leaf size, leaf number, biomass, and leaf area, as well as cell growth and elongation due to limited and insufficient water transport through the xylem and into surrounding cells (Bhandari et al., 2023). The formation of green spots is an early indicator of cellular activity during the process of callus regeneration into embryos, signifying that the callus is embryogenic. However, the presence of green spots does not always correlate positively with the number of plantlets formed. This discrepancy may be due to an imbalance between auxin and cytokinin growth regulators within and outside the cells (Lestari and Yunita, 2008). Both Cigeulis and IR64 varieties exhibited green spots under control and salinity treatments, with the IR64 variety showing a higher percentage of green spots compared to Cigeulis (Table 4). This finding suggests that both varieties can tolerate salinity stress up to a concentration of 150 mM. The differences in green spot formation between the two varieties highlight the role of genotype in influencing callus induction and regeneration under salinity stress (Khatun et al., 2003). This may be attributed to the fact that Cigeulis rice is a crossbreed of the IR64 variety (Mahaputra et al., 2024). Plants with robust growth are better able to absorb nutrients, enhancing metabolic activity and chlorophyll development within the callus and contributing to green spot formation (Kafisa et al., 2016). During regeneration, pre-embryogenic stages, including the globular, scutellar, coleoptilar, and plantlet phases, were observed. The globular stage marks the initial phase of somatic embryo development. During the subsequent scutellar stage, callus cells begin forming scutellar structures resembling embryonic tissue within seeds. At this stage, cells organize into more complex structures, and the embryo adopts an elongated or oval shape characteristic of the scutellar stage. Following this, the embryo forms more specialized structures, such as the coleoptile, which acts as a protective sheath for the developing shoot.

Embryogenic cells are characterized by their spherical shape, dense cytoplasm, and reactivity to acetocarmine, while non-embryogenic cells are vacuolar, elongated, and permeable to Evans blue (Guo et al., 2019). Acetocarmine reacts with embryogenic cells, staining them red on the callus, while Evans blue reacts with non-embryogenic cells, marking them blue (Khatri and Joshee, 2024). Cytochemical staining in the control treatment for both varieties resulted in nearly the entire callus body turning red (Fig. 3*A*), indicating the presence of embryogenic cells. In the 100 mM (P1) and 150 mM (P2) NaCl treatments, a color gradient was observed in both varieties, with the intensity of blue coloration increasing with higher NaCl concentrations. This gradient indicates that non-embryogenic cells, which absorb Evans blue, become more prominent as salinity stress increases. Embryogenic callus, as shown in Fig. 3*A*, exhibits a smooth, bright, globular structure with a dense and compact texture. The presence of globular structures signifies tolerance to applied stress or pressure. These structures are critical for the differentiation of embryogenic cells into various cell types, enabling the formation of complex plant structures. Embryogenic callus produces small somatic embryos capable of regenerating into complete plants, including roots and shoots, whereas non-embryogenic callus is fragile, watery, and lacks regenerative potential as shown in Fig. 4*A* (Takamori et al., 2015). The percentage of embryogenic callus decreased in both varieties as the salinity concentration increased (Fig. 3*B*).

The number of plantlets regenerated from the IR64 variety was higher than that of the Cigeulis variety under both control and salinity treatments. During the regeneration period, callus development into plantlets demonstrated that salinity stress caused stunted rice plant growth. Symptoms of salinity stress include stunted growth, leaf senescence, and leaf tips appearing burned or yellowed, as noted by Muharam and Saefudin (2016). Additionally, differences in root and leaf growth characteristics indicate that the IR64 variety possesses better regenerative ability compared to the Cigeulis variety. Salt-tolerant rice plants employ defense mechanisms involving antioxidant gene activity to detoxify reactive oxygen species (ROS) and protect cells from potential damage. Key ROS scavengers include superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), which are vital components of the plant's defense system. The expression of genes encoding APX, CAT, and SOD is modulated by environmental stressors, including salinity (Hasanuzzaman et al., 2021). SOD activity is a key indicator of plant sensitivity, as it serves as the first line of defense against ROS (Aazami et al., 2021). SOD neutralizes superoxide radicals by producing H_2O_2, which is subsequently removed by CAT. APX plays a crucial role in detoxifying H_2O_2, stabilizing membranes, and contributing to CO_2 fixation (Thamodharan et al., 2023).

In the Cigeulis variety, clearly expressed antioxidant genes include *OsCATA*, *CAT*, *MnSOD*, *Cu/ZnSOD*, *cytosolic APX*, *OsAPX1*, and *P5CS*, the gene encoding an enzyme for proline synthesis (Fig. 4*B*). These genes demonstrated increased band intensity with rising NaCl concentrations, reflecting enhanced gene expression under salinity stress. Similarly, in the IR64 variety, genes such as *OsCATA*, *MnSOD*, *Cu/ZnSOD*, *cytosolic APX*, *OsAPX1*,

and *P5CS* showed increased DNA band intensity. Proline promotes salinity stress tolerance and acts as an organic nitrogen storage during stress recovery (Aazami et al., 2021). Proline also stimulates the expression of salt-responsive proteins. The thicker DNA bands observed in both varieties suggest heightened gene expression in response to salinity stress. However, the genes *GPOD* and *GABA-T* were not clearly expressed in either variety, indicating variable gene responses to salinity treatment. These findings highlight that salinity influences the expression levels of genes encoding antioxidant enzymes and the accumulation of GABA and proline in rice plants, contributing to their defense mechanisms.

Conclusions

This study explores the effect of salinity stress on callus induction, regeneration, and gene expression in IR64 and Cigeulis rice varieties. Results show that IR64 exhibits superior tolerance to salinity, with higher callus induction, regeneration, and antioxidant gene expression compared to Cigeulis. Increased salinity concentrations led to reduced embryogenic callus formation, browning, and smaller callus size, with green spot formation correlating negatively with salt stress. Upregulation of antioxidant genes (*APX*, *CAT*, *SOD*) and pyrroline-5-carboxylate synthetase (P5CS), a primary metabolite-related gene (P5CS), suggests that these mechanisms help mitigate salt-induced damage in rice. Future research should focus on further characterizing the genes involved in salinity tolerance and their molecular interactions. Genome-wide association studies and *CRISPR*-Cas9 gene editing could identify key genes improving salt tolerance in rice.

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