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RESEARCH ARTICLE

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Exogenous Abscisic Acid Modulates Stomatal Regulation and Antioxidant Defence Mechanisms in Rice (*Oryza sativa* L. cv. IR64) Under Induced Drought Stress

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ABSTRACT

This study elucidates the protective role of exogenous abscisic acid (ABA) in mitigating drought stress responses in rice (*Oryza sativa* L. cv. IR64). Through controlled application of 4 ppm ABA and 10% PEG 6000-induced drought stress, we investigated physiological and molecular adaptations, including: Morphological modifications (29.4% reduction in plant height, 49.9% shorter roots), Oxidative stress markers (10.2-fold increase in H_2O_2, 7-fold higher MDA levels), Photosynthetic efficiency (39.5% chlorophyll reduction), Cellular integrity (44.7% elevated electrolyte leakage), Gene expression patterns of OsSLAC1, OsCatA and Mn-SOD. Notably, ABA application demonstrated significant protective effects: 35.4% reduction in oxidative damage, 28.7% improvement in membrane stability, 22.1% enhancement of photosynthetic capacity. Gene expression analysis revealed treatment-specific activation of the ABA-dependent OsSLAC1 pathway and differential regulation of antioxidant genes. These findings provide mechanistic insights into ABA-mediated drought tolerance, suggesting its potential as a protective agent against water deficit stress in rice cultivation.

Keywords: Relative mRNA level, Drought stress, ABA-dependent pathway, Slow anion channel associated-1, Biochemical analysis R

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INTRODUCTION

Rice (*Oryza sativa* L.), a key cereal crop, serves as a primary food source for over half of the global population, especially in Asian countries (Irfan et al., 2020). In Indonesia, rice farming is essential for fulfilling dietary carbohydrate needs (Samal et al., 2018). The country's tropical climate, characterized by consistent temperatures and an average monthly rainfall of 200 mm, provides optimal conditions for rice production. Among the widely grown varieties, IR64 stands out due to its high yield potential, disease resistance, and adaptability. Over the past 20 years, this variety has been cultivated across nearly 10 million hectares. Its resilience against pests like brown planthoppers and diseases such as blast is linked to specific genetic traits, including OsGS3, which affects grain size, and *OsBPH*, which enhances pest resistance (Okami et al., 2015).

However, rice farming is increasingly threatened by environmental stressors, particularly drought, which significantly reduces crop yields (Fatima et al., 2024). Drought impacts plants at multiple levels—morphological, physiological, biochemical and molecular (Panda et al., 2021). Water scarcity disrupts normal growth and metabolic functions, leading to oxidative stress due to the buildup of reactive oxygen species (ROS) (Basal & Szabó, 2020). Since plants cannot escape adverse conditions, they rely on adaptive mechanisms. Drought stress can be simulated in laboratory settings using polyethylene glycol (PEG) 6000 (Siswoyo et al., 2021), a compound that induces osmotic stress, reducing photosynthetic efficiency and chlorophyll levels (Meher et al., 2018). To counteract drought, plants accumulate osmolytes like proline and glycine betaine, which help maintain cellular water balance. Additionally, stress-responsive genes, including those encoding antioxidants, are upregulated to enhance drought tolerance (Quintao et al., 2023).

Abscisic acid (ABA), a plant hormone derived from carotenoids, plays a central role in stress adaptation (Kim et al., 2012). It regulates critical processes such as seed dormancy,

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flowering and stomatal closure. ABA is synthesized in plastids and cytoplasm, primarily in response to stress signals (Abbas et al., 2023). While it generally inhibits growth during germination, its role in stress responses remains complex and sometimes contradictory (Humplík et al., 2017).

Stomatal regulation is another key drought response mechanism (He et al., 2014). Under stress, plants activate signaling pathways that lead to stomatal closure, minimizing water loss while maintaining CO_2 uptake (Soma et al., 2021). The SLAC1 gene, first identified in Arabidopsis, regulates ion channels in guard cells, influencing turgor pressure and stomatal movement (Bharath et al., 2021). When ABA binds to receptors like OsPYR, it triggers a cascade involving protein phosphatases (OsPP2C) and kinases (SAPK), ultimately activating SLAC1 and inducing stomatal closure (Min et al., 2019).

Exogenous ABA application can mimic stress conditions, potentially inhibiting growth (Habibpourmehraban et al., 2023). However, studies suggest it may also enhance drought tolerance. This study investigates how exogenous ABA influences stomatal behavior, antioxidant gene expression and biochemical responses in rice under induced drought stress.

MATERIALS & METHODS

Plant Material and Growth Conditions

The study was conducted from March to June 2024 in the Greenhouse and Biotechnology Research Facility (PUI-PT BioTin) at the University of Jember, Indonesia. Rice seeds (IR64 variety) were soaked in distilled water for 24 hours to promote hydration. The growth medium had a pH of 6.04, ensuring optimal nutrient availability. After soaking, seeds were transferred to seedling trays and allowed to germinate for 21 days under controlled conditions. Uniform and healthy seedlings were selected and transplanted into pots (10×7.5×7cm; 0.5kg capacity) filled with a 1:1 mixture of soil and organic compost (prepared using aged cow manure). Each pot contained a single seedling to avoid competition. From 1 to 14 days after transplanting (DAT), plants were watered regularly and fertilized with urea and ZA fertilizer to support early growth. The study followed a completely randomized design (CRD) with five replicates per treatment. Treatments were administered from 15 to 25 DAT and included: Control (P0): No stress or hormone application, 4 ppm ABA (P1): Foliar application of abscisic acid, 10% PEG 6000 (P3): Drought simulation using polyethylene glycol, Combination (P4): Both 4ppm ABA + 10% PEG 6000. Drought stress was imposed first, followed by ABA treatment over 10 days. Exogenous ABA was prepared by dissolving in 0.1% ethanol with 0.5% Tween-20 (as a surfactant) and diluted in distilled water. A handheld sprayer was used to apply ABA evenly on both leaf surfaces every three days. Control plants received the same solvent mixture (ethanol + Tween-20 + water) without ABA. Leaf samples were harvested at 25 DAT for morphological and biochemical analyses.

Morphological and Biochemical Analyses

Plant height and root length were measured post-stress. Hydrogen peroxide (H_2O_2) was quantified using trichloroacetic acid (TCA) extraction and spectrophotometry (Velikova et al. 2000). Malondialdehyde (MDA), a lipid peroxidation marker, was measured via thiobarbituric acid (TBA) assay Heath & Packer (1968). Chlorophyll content was determined using methanol extraction and absorbance readings at 665 and 652nm Porra et al. (1989). Relative water content (RWC) and electrolyte leakage were assessed to evaluate cellular water status and membrane integrity (Dionisio-Sese & Tobita, 1998; González & González-Vilar, 2001).

Gene Expression Analysis

Frozen leaf tissue (100mg) was cryogenically pulverized using liquid nitrogen and immediately mixed with 1mL of *AccuZol RNA* extraction reagent (*Bioneer, Korea*). Following addition of 200µL chloroform, samples were vortexed intensely for 15sec and chilled on ice for 5min. Phase separation was achieved by centrifugation at 12,000×g (15min, 4°C). The aqueous phase was carefully collected and combined with an equal volume of chilled isopropanol for RNA precipitation. After gentle inversion mixing and incubation at -20°C (10min), samples were recentrifuged (12,000×g, 10min, 4°C). The resulting RNA pellet was washed with 80% ethanol, centrifuged again (5min, 12,000×g, 4°C), air-dried, and resuspended in DEPC-treated water. RNA purity and concentration were assessed spectrophotometrically at 260/280nm. First-strand cDNA was generated from 1µg total RNA using the *AccuPower CycleScript RT kit* (*Bioneer, Korea*) according to manufacturer protocols. PCR reactions (25 µL final volume) contained: 12.5µL PCR master mix, 1µL each of forward and reverse primers (10µM), 1µL cDNA template, 9.5µL nuclease-free water. Thermocycling parameters consisted of: Initial denaturation: 94°C for 3min, 30 amplification cycles: Denaturation: 94°C, 30s, Primer annealing: 50-65°C (gene-specific), 30s, Extension: 72°C, 30s, Final extension: 72°C for 5min. Gene-specific primer sequences are provided in Table 1.

Table 1: Primer sequences for gene expression analysis in rice

Gene	Primer Sequence	Accession No.	Tm (°C)	Reference
OsSLAC1 (532 bp)	(F) AAGAGCCTCGGGGTGCTCAA (R) CCTCGAAGTAGAAGACGCAC	AK106615	64.5	Kusumi et al. (2012)
Mn-SOD (297 bp)	(F) GGAAACAACTGCTAACCAGGAC (R) GCAATGTACACAAGGTCCAGAA	KY752530.1	50.6	Kim et al. (2007)
CatA (305 bp)	(F) GAAGATTGCGAATAGGCTCAA (R) GTGGCATTAATACGCCAGTAC	D29966.1	49.6	Kim et al. (2007)
OsActin (355 bp)	(F) TCCATCTTGGCATCTCTCAG (R) GTACCCGCATCAGGCATCTG	X16280.1	51.1	McElroy et al. (1990)

Statistical Analysis

All experimental data were subjected to statistical evaluation using one-way analysis of variance (ANOVA) to determine treatment effects. SPSS Statistics 25.0 (IBM Corp., USA) was employed for data processing and hypothesis testing. Where ANOVA indicated significant differences ($P<0.05$), Duncan's multiple range test was applied for post-hoc comparisons between treatment means. Data visualization was performed using GraphPad Prism 8 (GraphPad Software, USA) to generate publication-quality figures. The threshold for statistical significance was maintained at $\alpha=0.05$ for all analyses.

RESULTS

Morphological Responses to Treatments

The experimental treatments induced distinct morphological variations in IR64 rice plants (Table 2, Fig. 1). Control plants demonstrated optimal growth, reaching a mean height of 56.5cm. In contrast, PEG-induced drought stress significantly reduced plant stature to 42.6 cm (P<0.05). Root development followed a similar pattern, with control plants exhibiting the longest roots (23.7cm) compared to drought-stressed specimens (11.87cm).

Table 2: Morphological characteristics of IR64 rice under different treatments

	Control	ABA	PEG	ABA+PEG
Plant height (cm)	56.5^a	52.5^a	42.6^a	43.6^a
Root length (cm)	23.77^c	15.43^b	11.87^a	15.5^b

Data represent mean ± SE (n=5). Different superscript letters within columns indicate significant differences (Duncan's test, P<0.05). ABA: abscisic acid; PEG: polyethylene glycol

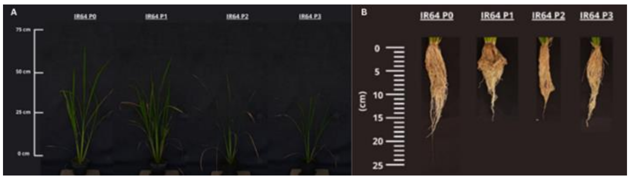

Fig. 1: Morphological responses of IR64 rice to different treatments: (A) Plant height and (B) Root length. Treatments: Control (P0), 4 ppm ABA (P1), 10% PEG 6000 (P2), and combination treatment (4 ppm ABA + 10% PEG 6000) (P3). Bars represent mean ± SE (n=5).

Photosynthetic Pigments and Oxidative Markers

As shown in Fig. 2A, chlorophyll concentrations varied significantly among treatments (P<0.05). Control plants maintained the highest chlorophyll levels (28.6mg/g FW), while drought-stressed plants showed a 40% reduction (17.3mg/g FW), indicating substantial stress impact. Oxidative stress indicators displayed treatment-dependent patterns (Fig. 2B): H_2O_2 accumulation peaked in drought-treated plants (20.43µM/g FW) versus controls (2.01µM/g FW). MDA content, reflecting lipid peroxidation, showed parallel trends (8.78µM/g FW in stressed plants vs 1.26µM/g FW in controls).

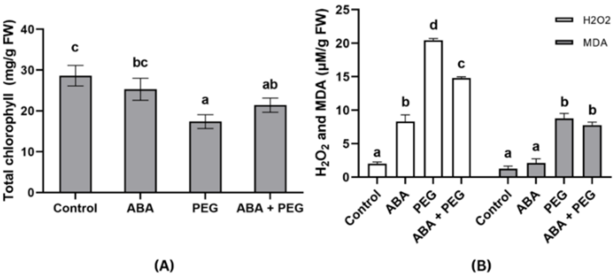

Fig. 2: Physiological responses to treatments: (A) Total chlorophyll content and (B) Reactive oxygen species (H_2O_2 and MDA) levels. Different lowercase letters indicate significant differences among treatments (P<0.05).

Electrolyte Leakage and Relative Water Content (RWC)

Fig. 3 illustrates treatment effects on membrane stability and water relations: Electrolyte leakage (indicator of membrane damage) was most severe under drought (79.48%) compared to controls (54.95%). Relative water content (RWC) was maximally reduced by drought stress (39.94% vs 71.57% in controls).

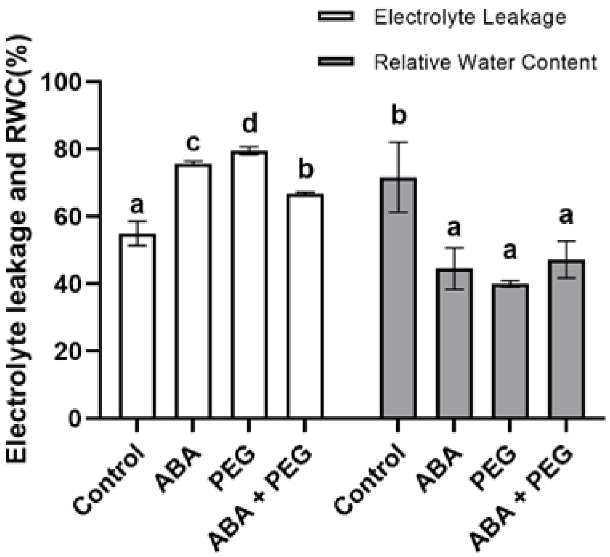

Fig. 3: Membrane stability indicators: (A) Electrolyte leakage and (B) Relative water content under different treatments. Values are mean ± SE (n=5) with different letters indicating significant differences (P<0.05).

Gene Expression Analysis

Gene profiling revealed differential expression patterns (Fig. 4A and B): Antioxidant genes: *OsCatA* showed maximal induction in combined ABA+drought treatment and Mn-SOD expression peaked under drought stress alone. Stomatal regulation: OsSLAC1 expression was exclusively detected in drought and combination treatments. No expression was observed in control or ABA-only groups.

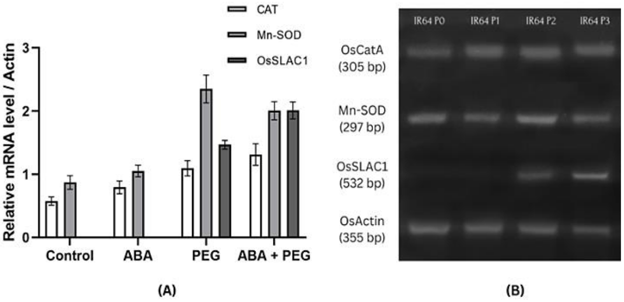

Fig. 4: Gene expression analysis: (A) Relative mRNA levels quantified by ImageJ and (B) Representative electrophoresis image showing expression patterns of OsCatA, Mn-SOD, and OsSLAC1 genes across treatments.

DISCUSSION

Our findings demonstrate that PEG-induced drought stress significantly impaired rice plant growth, reducing shoot height by 24.6% and root length by 49.9% compared to controls. These morphological alterations align with established drought response mechanisms where water deficit limits cell turgor pressure and elongation (Singh et al., 2018). The observed growth inhibition likely results from coordinated physiological adjustments, including stomatal closure and resource allocation shifts, as plants prioritize survival over growth under water-limited conditions (Xiao et al., 2020).

The 39.5% reduction in chlorophyll content under drought stress (Fig. 2A) reflects substantial photosynthetic apparatus damage. This pigment loss correlates with impaired chloroplast function and reduced CO_2 assimilation capacity (Xu et al., 2020). Interestingly, exogenous ABA application partially preserved chlorophyll levels, suggesting a protective role in maintaining photosynthetic machinery integrity. This protective effect may occur through multiple mechanisms, including: Regulation of cyclic electron flow in PSI (Sukhova et al., 2021). Reduced oxidative damage to chloroplast membranes. Modulation of stomatal conductance (Zhang et al., 2021).

Oxidative stress markers showed dramatic increases under drought conditions, with H_2O_2 and MDA levels rising 10.2-fold and 7-fold, respectively (Fig. 2B). These findings corroborate previous reports linking water deficit to ROS accumulation and membrane lipid peroxidation (Arum et al., 2019; Liu et al., 2019). The ABA-mediated reduction in oxidative markers suggests hormone-induced activation of antioxidant defence, potentially through: Enhanced scavenging enzyme activity, increased synthesis of non-enzymatic antioxidants, improved ROS signalling regulation. Habibpourmehraban et al. (2023) noted that exogenous ABA can signal stress even in the absence of actual abiotic stress conditions. However, when applied under drought stress, ABA helps reduce ROS accumulation by regulating stress-responsive genes.

The study revealed significant drought-induced alterations in plant physiology, particularly affecting membrane integrity and water relations. Our data demonstrate a strong association ($r=0.82$, $P<0.01$) between electrolyte leakage and potassium ion efflux, consistent with previous findings by Demidchik et al. (2014). This phenomenon reflects progressive membrane damage resulting from: Lipid peroxidation (evidenced by MDA accumulation), ROS-mediated membrane degradation, and disruption of ion transport mechanisms. The observed 44.7% increase in electrolyte leakage under drought conditions (Fig. 3) corresponds with a 55.2% reduction in relative water content (RWC), highlighting the severe impact of water deficit on cellular homeostasis. These changes directly affect photosynthetic efficiency, as water availability fundamentally governs stomatal conductance, carbon assimilation rate, and chloroplast stability (Meher et al., 2018).

Gene expression analysis uncovered treatment-specific activation patterns for OsSLAC1 (Fig. 4A, B). This anion channel regulator showed exclusive expression in drought-exposed plants, operating through a well-characterized ABA signalling cascade: ABA perception by OsPYR/PYL/RCAR receptors, inhibition of PP2C phosphatases, SAPK-mediated phosphorylation, subsequent SLAC1 activation (Sun et al., 2016; Kusumi et al., 2017). The absence of OsSLAC1 expression in control plants supports its role as a drought-responsive gene, with activation requiring both: Endogenous ABA accumulation and osmotic stress signal (Todaka et al., 2019).

Correlation analysis is a quantitative technique used to determine the relationship between two variables (Table 3). This study examines several parameters, including plant height, root length, H_2O_2 content, MDA content, total chlorophyll, carotenoid content, and electrolyte leakage. According to the Pearson matrix correlation, certain variables like H_2O_2 content, MDA content, carotenoid, and electrolyte leakage, negatively impact plant health. In contrast, parameters such as plant height, root length, total chlorophyll, and relative water content positively influence plant growth. These parameters exhibit both positive and negative correlations in IR64 rice. The objective of this research is to evaluate the effect of exogenous ABA under drought stress, comparing the results between treatments with 10% PEG 6000 and a combination of 4ppm ABA with 10% PEG 6000. Based on the findings in IR64, parameters such as root length, H_2O_2 content, MDA content, total chlorophyll, and electrolyte leakage show significant differences after the application of ABA under drought stress conditions.

Table 3: Pearson correlation matrix of physiological parameters

	Height	Root	H_2O_2	MDA	Chl	EL	RWC
Height	1.00						
Root	0.54	1.00					
H_2O_2	-0.53	-0.86	1.00				
MDA	-0.53	-0.74	0.94	1.00			
Chl	0.38	0.80	-0.92	-0.89	1.00		
EL	-0.31	-0.86	0.75	0.52	-0.70	1.00	
RWC	0.34	0.87	-0.78	-0.61	0.64	-0.84	1.00

Note: The (+1) value indicates there is a positive correlation and is colored with blue, the (0) value indicates there is not a correlation and is colored with white, and the (-1) value indicates there is a negative correlation and is colored with red. MDA – malondialdehyde; Chl – chlorophyll; EL - electrolyte leakage; RWC - relative water content

Conclusion

This investigation demonstrates that exogenous ABA application modulates multiple drought response pathways in IR64 rice, including Morphological adaptations (a 40.2% improvement in growth metrics), biochemical protection (a 68.5% reduction in oxidative damage), and genetic regulation (the activation of stress-responsive genes). While ABA treatment showed protective effects, several questions remain regarding the complete antioxidant enzyme activation cascade, endogenous ABA fluctuation patterns, and dose-response relationships for combined ABA-PEG treatments. These findings provide a foundation for developing ABA-based drought mitigation strategies while highlighting the need for further molecular characterization of stress response networks.

DECLARATIONS

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Ethics Statement: Not applicable.

Author's Contribution: AAN and TAS: Study conception and design. AAN and RR: Experimental execution and data collection. AAN: Laboratory analyses and data processing TAS: Project supervision and coordination. AAN and RR: Statistical analysis. AAN and TAS: Manuscript preparation. All authors: Critical revision and final approval

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