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Acclimation to drought stress improves root physiology and cell mitotic index, leave pigments and water status in *Oryza sativa* L.

Abstract. Drought is a prominent abiotic stressor that critically impairs rice productivity by disrupting fundamental physiological processes and diminishing yield potential. This study investigates the effects of continuous and alternating drought stress on cell mitotic index and physiological responses in selected traditional and domestically grown rice varieties. Drought stress was imposed for approximately 9 days, a condition visibly marked by the rolling of leaves. In the alternating drought treatment, plants were exposed to identical drought conditions but were rehydrated to normal water levels on the 10th day, with this cycle of drought and rehydration repeated twice. Continuous drought stress led to a significant reduction in root cell mitotic activity, with a decrease ranging from 23.6% to 67% compared to control conditions. Additionally, drought stress adversely impacted leaf physiology, evidenced by reductions in total chlorophyll content, Fv/Fm ratios, and relative leaf water status. In contrast, the severity of these physiological disruptions was less pronounced in plants subjected to alternating drought stress. In addition, while acclimated plants exhibited elevated levels of root electrolyte leakage (REL) and malondialdehyde (MDA) compared to controls, these levels were substantially lower than those observed under continuous drought stress. These findings suggest that while drought negatively affects most physiological parameters, its impact can be mitigated through intermittent watering and the use of drought-tolerant rice varieties.

Key words: Rice, drought stress, mitotic index, root electrolyte leakage, malondialdehyde.

Introduction

Rice or *Oryza sativa* L., is known to be one of the most consumed staple foods worldwide [1]. It plays a critical role in ensuring food security, preserving cultural heritage, and shaping governmental policies in developing nations [2,3]. Projections anticipate that the world population will reach approximately 9.8 billion by 2050, leading to a projected doubling in demand for rice [4]. Presently, rice constitutes a primary dietary staple for about half of the world's population, with Asia contributing nearly 90% to its global production. The required volume of rice to meet future demands has escalated by 70% due to the expanding population in Asia [5]. Despite these trends, approximately 35% of rice-growing regions worldwide are experiencing production stagnation [6,7].

Meanwhile, plants inherently fixed in the soil, are inevitably exposed to a wide range of potentially detrimental environmental conditions, including drought stress [8]. Drought, characterized by a prolonged period of water scarcity leading to soil moisture falling below saturation levels to completely convey the yield capacity [9,10]. Globally, drought substantially reduces cereal production by 9 to 10%, profoundly impacting plant growth, physiology, and grain development [11-14]. Drought stands as the foremost factor affecting agricultural productivity and thereby poses a significant threat to global food security [15,16].

Plant growth is primarily driven by processes such as cell division, cell growth, and differentiation. Drought stress, like other abiotic stressors, is reported to suppress the mitotic index [17,18], resulting in compromised growth due to impaired cell elongation and mitosis [19]. The lack of turgor pressure under drought conditions significantly hinders cell growth and development, making cell growth one of the most vulnerable physiological processes to drought [19-21]. Even mild drought stress has been suggested to negatively affect cellular development, potentially leading to cell death under prolonged and severe drought conditions. This disturbance in metabolism further impacts physiological functions [22,23].

Drought conditions are classified into two primary types: intermittent and terminal [24]. Terminal drought is characterized by a significant reduction in the water available for plant uptake, leading to severe drought stress and ultimately resulting in plant mortality. In contrast, intermittent drought conditions, which may occur either in discrete episodes or repeatedly throughout the growing season, adversely affect plant development when there is insufficient irrigation or rainfall. Unlike terminal drought, intermittent drought is typically not lethal. The capacity of plants to survive and maintain functionality under both intermittent and extreme drought conditions is indicative of their drought tolerance or resistance mechanisms [25]. Therefore, the aim of this study was to investigate the effects of two different types of drought, continuous and alternating, on the root mitotic activity and physiological responses of rice plants.

Materials and methods

Field preparation and treatments. This study was conducted at the Department of Plant Science, International Islamic University Malaysia with six varieties; UPM Putra 2, Kuku Belang, Jarum Mas, Dular, Huma Wangi Lenggong (HWL) and a homozygous breeding Line 541 (L541) were compared to a susceptible but domestically grown MR297. Rice seedlings were cultivated in 9 cm x 12 cm polybags containing 100% topsoil. The polybags were then placed in containers and subsequently filled with water to submerge the basal part of the plants. All plants were organized following to a completely randomised design with five replications for each treatment. Throughout the experiments, standard rice cultivation practices, including fertilization, were followed. After 28 DAS, drought stress was initiated by withholding water for approximately 9 days, indicated by the appearance of rolled leaves in plants. Meanwhile, the alternate drought treatment, was implemented under identical conditions, with the exception that plants were rehydrated back to normal water levels on day 10. This cycle of alternating drought was done twice.

Mitotic index analysis. Nuclei were isolated from the terminal 1.0 mm segment of root tips using a sharp sterile scalpel blade. The chopping procedure was performed in 0.8 ml of a slightly modified LBOI buffer (15 mM Tris, 2 mM EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM spermine, 15 mM mercaptoethanol, 0.1% Triton X-100, pH 7.5), following the method described by [26]. The suspension was subsequently filtered through a 40 µm cell strainer. Post-filtration, 50 µl of Propidium Iodide (PI) was added for DNA staining, along with 50 µl of RNase, and allowed to incubate for 10 minutes. The nuclei suspension was then analyzed using a Muse® Cell Analyzer flow cytometer. The percentage of cells in the G2/M phase was determined from the data extracted from the Muse® Cell Analyzer (Merck Millipore, Darmstadt, Germany).

Measurement of chlorophyll fluorescence. Chlorophyll fluorescence was measured on a fully grown leaf using a portable chlorophyll fluorometer PAM-2500 (Heinz Walz GmbH). Prior to measurements, the leaf was subjected to dark adaptation for approximately 30 minutes. The ratio of variable to maximum fluorescence (Fv / Fm), indicative of the maximum quantum yield of Photosystem II (PS II), was recorded following the method described by [27].

Leaf total chlorophylls and carotenoids content. Total chlorophyll content was determined following the procedures by [28] with minor adjustments. A known weight of fresh leaves was homogenized in 3 ml of 80% acetone using a mortar and pestle. The extracts were then centrifuged at 13.3g for 5 minutes, and the supernatants were collected. Total chlorophylls were quantified using a spectrophotometer at 663.6 nm, 646.6 nm, and 750 nm.

Meanwhile, carotenoid content was analysed by homogenizing a known weight of fresh leaves with 3 ml of 80% acetone following the method by [29]. The pigment concentrations were determined spectrophotometrically at 645 nm, 663 nm, and 480 nm with 80% acetone used as blank.

Relative water content (RWC). Leaf relative water content (RWC) was assessed using a known weight of fresh leaves. The samples were hydrated in distilled water for 4 hours in a petri dish to reach a maximum turgor capacity. Subsequently, the leaves were gently dried with tissue paper before measuring their turgor weight. Following this, the samples were dried in an oven at 60°C for three days until a constant weight was achieved for leaf dry weight (DW).

Measurement of root electrolyte leakage (REL). A modified method measuring REL on fine roots was employed based on the procedures by [30-32]. Root samples weighing between 100 and 500 mg were placed in 16 ml of distilled or deionized water within 28-ml universal glass bottles, achieving a tissue-to-water ratio of approximately 1:20. The bottles were sealed, shaken continuously, and incubated at room temperature for 12 hours. The initial conductivity (C1) of the solution was measured using an electrical conductivity (EC) meter. Subsequently, the samples were autoclaved at 110°C for 10 minutes, cooled to room temperature, and the second conductivity measurement (C2) was recorded. REL was calculated using the following formula:

$$\mathrm{REL} = (C_1/C_2) \times 100$$

Measurement of lipid peroxidation. Lipid peroxidation was assessed by measuring malondialdehyde (MDA) production following the protoco by [33] with some modifications. Approximately 0.2-0.4 g fresh weight of roots were homogenised in 4 ml of 0.1% (w/v) trichloroacetic acid (TCA, followed by centrifugation at 10 000 G for 10 minutes. A 750 µL aliquot was taken and mixed vigorously with same amount of (A) +TBA solution containing 20% (w/v) TCA and 0.5% (w/v) thiobarbituric acid (TBA), and (B) –TBA containing only 20% (w/v) TCA. The samples were then heated at 95˚C for 25 minutes in a water bath and subsequently cooled down to room temperature prior to centrifugation at 10 000 G for 10 minutes. The absorbance at 440, 532 and 600 nm were recorded. MDA in each sample was calculated as follows:

$$(\mathrm{Abs}\ 532\ +\mathrm{TBA} - \mathrm{Abs}\ 600\ +\mathrm{TBA}) -$$
$$- (\mathrm{Abs}\ 532\ -\mathrm{TBA} - \mathrm{Abs}\ 600\ -\mathrm{TBA}) = A$$
$$(\mathrm{Abs}\ 440\ +\mathrm{TBA} - \mathrm{Abs}\ 600\ +\mathrm{TBA}) \times 0.0571 = B$$
$$\mathrm{MDA}\ (\mathrm{nmol.mL^{-1}}) = [(A\text{-}B) / 157000] * 1\mathrm{x}10^6$$

Statistical analysis. All the data were analyzed using GraphPad Prism Version 9 and one-way ANOVA was performed to test significance differences ($p \leq 0.05$) among the treatment means. The overall effects of different paddy varieties and drought treatments and their interactions on growth, physiological and biochemical parameters were determined by comparing treatments means with a two-way ANOVA at $p \leq 0.05$.

Results and discussion

Effect of drought and alternate drought on roots mitotic activity. The present study aimed to assess the resilience of root cell cycle dynamics under drought and alternating drought conditions (Figure 1).

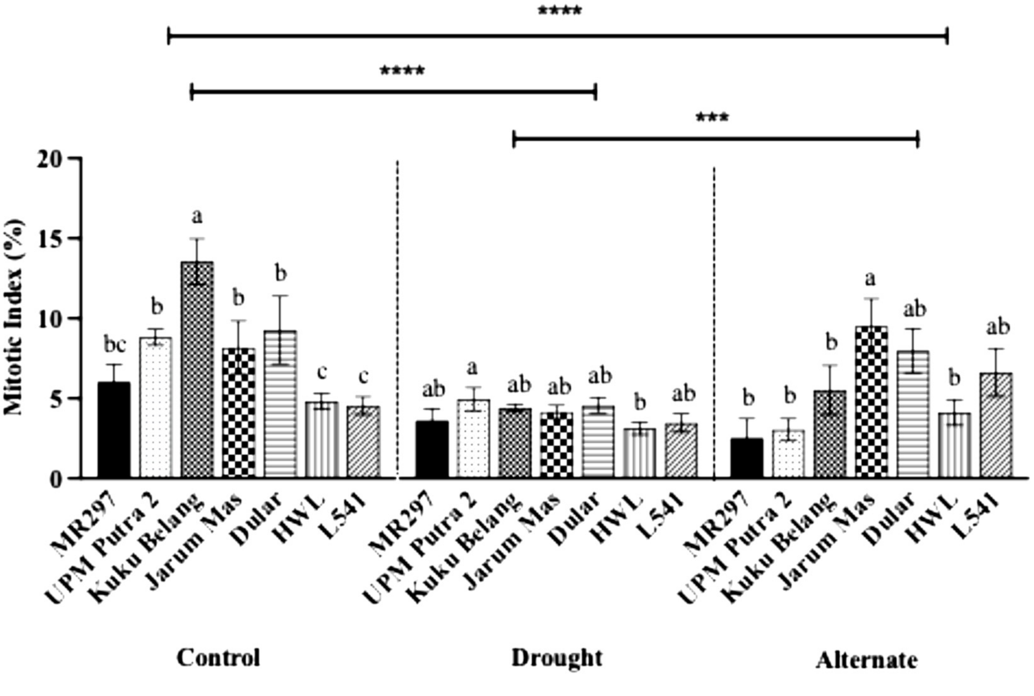

Figure 1 – Mitotic index in rice seedlings subjected to normal irrigation, drought and alternate drought stress. Data was analysed using one way ANOVA to assess the significance level between means ± SE of n = 5 ($p \leq 0.05$). Comparison was done amongst varieties within the same treatment and values are not sharing common letters differ significantly. Meanwhile, values are not sharing a common superscript (*, **, ***) differ significantly at $p \leq 0.05$, $p \leq 0.001$ and $p \leq 0.001$ (LSD), respectively

In the control condition, it was assumed that mitotic activities would align with the inherent characteristics of respective plant varieties. Notably, the Kuku Belang cultivar exhibited the highest mitotic activity at 13.6%, followed significantly by MR297, UPM Putra 2, Jarum Mas, Dular, HWL, and L541. However, under drought conditions, a significant reduction in mitotic activity was observed across all examined varieties. This reduction displayed variability among varieties with 39.8%, 43.9%, 67.5%, 49.2%, 50.7%, 35.2%, and 23.6% reduction compared to their respective control conditions. Further analysis also shows that alternating drought treatment yielded higher root mitotic activity in certain varieties than in continuous drought, particularly in Jarum Mas and Dular.

The regulation of the cell cycle is intricately dependent on a complex interplay of intrinsic and extrinsic factors, including hormonal signaling and environmental cues [34]. Previous research by [35] demonstrated that water stress led to a substantial reduction, approximately 90%, in endosperm cell division, accompanied by decreased thymidine incorporation into DNA and a concurrent attenuation of the endoreduplication process. This investigation suggests that certain rice varieties subjected to alternating drought conditions exhibit enhanced mitotic activity compared to those under continuous drought, indicating potential acclimation mechanisms that increase a plant's resilience to drought-induced stress, as evidenced by cellular mitotic activity.

Effect of drought and alternate drought stress on the leaf photosynthetic components. Changes in leaf physiology serve as key indicators of a plant's response to environmental conditions, particularly evidenced by alterations in photosynthetic-related components such as proteins and molecules during abiotic stress [36]. One crucial metric for assessing the impact of drought stress is the ratio of variable to maximum fluorescence, Fv/Fm, which represents the maximum photochemical quantum yield of photosystem II (PSII) under the theoretical condition where all reaction centers are open [37]. In the present investigation, plants subjected to controlled irrigation consistently exhibited Fv/Fm values exceeding 0.8, indicating the absence of stress-induced effects (Table 1).

Table 1 – Comparison of Fv/Fm, total chlorophyll and carotenoid content in different variety of rice within the same treatment subjected to drought and alternate drought stress. Data was analysed using a one-way ANOVA followed by LSD's post hoc test to assess the significance between means ($p \leq 0.05$). Values represent the mean ±SE of n = 4

	Control	Drought	Alternate drought
		Fv/Fm	
MR297	0.805^{b}	0.7432^{b}	0.7590^{c}
UPM Putra 2	0.808^{b}	0.7692^{a}	0.7680^{b}
Kuku Belang	0.814^{a}	0.7646^{a}	0.7964^{a}
Jarum Mas	0.810^{ab}	0.7682^{a}	0.7950^{a}
Dular	0.802^{b}	0.7714^{a}	0.7766^{b}
HWL	0.803^{b}	0.7682^{a}	0.7808^{b}
L541	0.807^{b}	0.7716^{a}	0.7914^{a}
	Total chlorophyll content (?		
MR297	1.46^{ab}	0.59^{b}	1.27^{b}
UPM Putra 2	1.74^{a}	0.47^{b}	1.37^{b}
Kuku Belang	1.36^{ab}	0.89^{a}	2.14^{a}
Jarum Mas	1.89^{a}	0.66^{a}	2.03^{a}
Dular	1.55^{a}	0.71^{a}	1.57^{ab}

Continuation of the table

	Control	Drought	Alternate drought
HWL	0.92^{b}	0.67^{a}	1.89^{a}
L541	1.76^{a}	0.36^{b}	1.92^{a}
	Carotenoid content (ug/ml)		
MR297	8.94^{a}	16.54^{c}	12.40^{c}
UPM Putra 2	5.96^{b}	15.12^{c}	8.96^{c}
Kuku Belang	5.92^{b}	39.78^{a}	18.28^{b}
Jarum Mas	9.97^{a}	20.72^{bc}	26.82^{a}
Dular	6.96^{ab}	20.95^{bc}	16.08^{b}
HWL	6.40^{ab}	24.47^{b}	13.84^{bc}
L541	4.82^{b}	17.48^{bc}	15.11^{b}

However, under drought stress conditions, all plants recorded values below 0.8, with MR297 showing the highest susceptibility compared to other varieties. Conversely, under alternating drought conditions, Kuku Belang, Jarum Mas, and L541 exhibited significantly higher Fv/Fm values, approaching 0.8. Despite being commercially cultivated, MR297 demonstrated the lowest Fv/Fm value. It is worth noting that Fv/Fm values in the range of 0.79 to 0.84 represent the approximate optimal range for many plant species, with lower values indicating photoinhibition of PSII and subsequent reduction in photosynthetic activity [38, 27]. According to [39], F_v/F_m in rice was severely affected at panicle initiation, flowering and ripening stages under drought condition. Meanwhile, according to [40] there was a positive correlation between Fv/Fm and drought.

The analysis of chlorophyll pigments demonstrated significant variations in the total chlorophyll content across different rice varieties. Under controlled irrigation conditions, Jarum Mas exhibited the highest natural chlorophyll content, followed sequentially by UPM Putra and Dular. However, the imposition of drought stress resulted in a reduction of chlorophyll content across all varieties, with MR297, UPM Putra 2, and L541 experiencing particularly greater decreases compared to Kuku Belang, Jarum Mas, Dular, and HWL. Interestingly, except for MR297 and UPM Putra 2, all plants showed a significant increase in total chlorophyll pigments under alternating drought conditions compared to their respective controls.

Chlorophyll is a critical component of the photosynthetic process, with the photosynthetic rate being shown to have a linear correlation with chlorophyll content [41]. The decline in chlorophyll levels is primarily attributed to the overproduction of reactive oxygen species (O2- and H2O2), which can induce lipid peroxidation of the chloroplast membrane during drought conditions. This lipid peroxidation impairs membrane stability and leads to the degradation of chlorophylls [42]. Additionally, abiotic stressors can cause damage to the chloroplast ultrastructure, resulting in reduced chlorophyll levels and consequently diminished photosynthetic activity [43].

The impact of continuous and alternate drought on carotenoid content was assessed and presented in Table 1. The carotenoid concentration was observed to be at its lowest under normal irrigation conditions. However, a significant increase in concentration was recorded in all plants following continuous drought exposure, in comparison to their respective varieties under control and alternate drought conditions. Previously, improved Fv/Fm ratios were attributed in part to efficient non-photochemical quenching (NPQ) mechanisms [45].

However, correlation analyses conducted in this study between Fv/Fm and both chlorophylls and carotenoid content revealed no significant differences. In contrast, a significant positive correlation was found between total chlorophylls and carotenoid content (r = 0.5885****). This suggests that the efficient dissipation of excess energy by carotenoids may contribute to the preservation of chlorophylls, particularly chlorophyll a, from degradation [44].

Effect of drought and alternate drought on leaf relative water content. Irrigation treatments was observed to have affected the relative water status in plants (Figure 2).

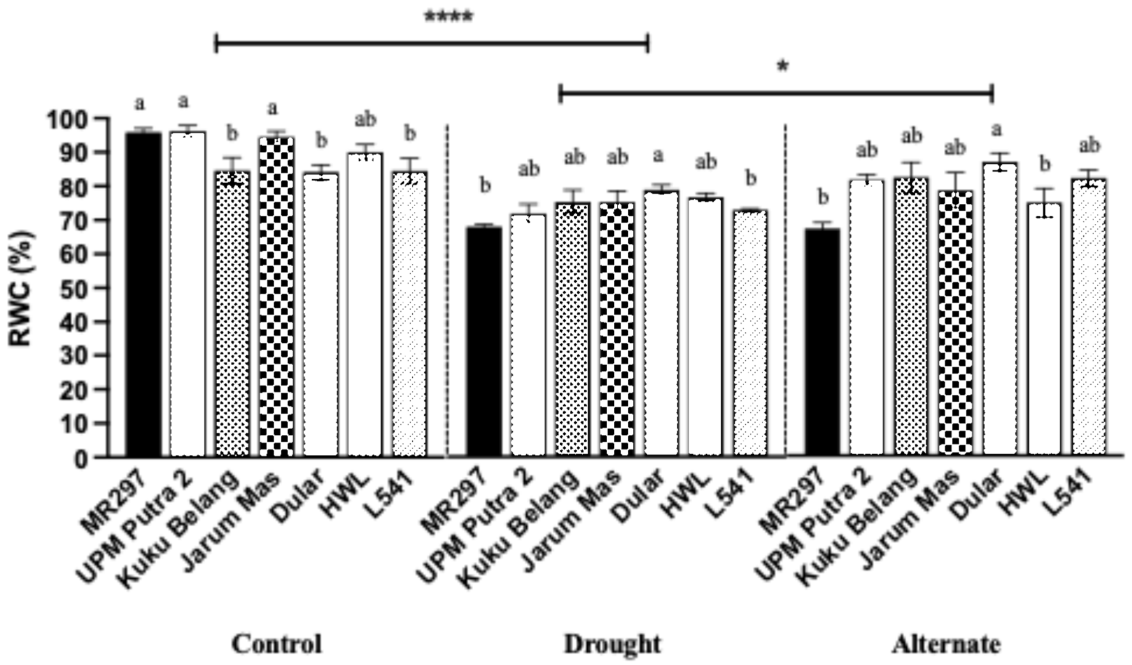

Figure 2 – Relative water content in rice seedlings subjected to normal irrigation, drought and alternate drought stress. Data was analysed using one way ANOVA to assess the significance level between means ± SE of n = 5 ($p \leq 0.05$). Comparison was done amongst varieties within the same treatment and values are not sharing common letters differ significantly. Meanwhile, values are not sharing a common superscript (*, **, ***) differ significantly at $p \leq 0.05$, $p \leq 0.001$ and $p \leq 0.001$ (LSD), respectively.

Under control irrigation conditions, all plants exhibited RWC values above 80%, indicative of normal leaf water status. In contrast, both continuous ($p \leq 0.0001$) and alternate drought treatments ($p \leq 0.001$) led to a significant reduction in RWC. Among the varieties studied, MR297 consistently displayed the lowest RWC under both drought conditions. Notably, plants subjected to alternate drought showed higher RWC compared to those under continuous drought stress. Drought stress typically results in a decrease in leaf RWC, often ranging from 60% to 80% [45]. However, the severity of this reduction depends on the plant's ability to mitigate stress through physiological adaptations such as stomatal regulation, acclimation in photosynthesis, and the accumulation of osmoprotectants to enhance cellular water conservation [38,46]. Acclimated plants do not only maintain higher RWC under drought stress but also exhibit resilience under other abiotic stress such as salinity and high temperatures [47,48,49]. In addition, studies also found higher accumulation of osmolytes including increased levels of amino acids, sugars, and ions as part of acclimation process [50,51]. The accumulation of these osmolytes was positively correlated with RWC and is associated with enhanced drought tolerance in plants [46].

*Effect of drought and alternate drought on root physiology***.** Drought stress significantly increased MDA levels in rice compared to both control conditions and alternate drought stress ($p \leq 0.001$***), with the highest accumulation observed in MR297 (Figure 3).

Although plants subjected to alternate drought exhibited higher MDA content compared to the control, the severity was significantly lower than that observed in continuously drought-stressed plants. This suggests that exposing rice plants to a sequence of drought events may enhance the physiological resilience of roots against the adverse effects of drought stress. MDA is a byproduct of lipid peroxidation caused by increased reactive oxygen species under stress condition [52,53]. This oxidative damage can lead to alterations in membrane-associated proteins, ionic channels, or receptors, thereby affecting their functionality [54]. Additionally, the release of phospholipid degradation enzymes, such as phospholipases, may further compromise membrane integrity [55].

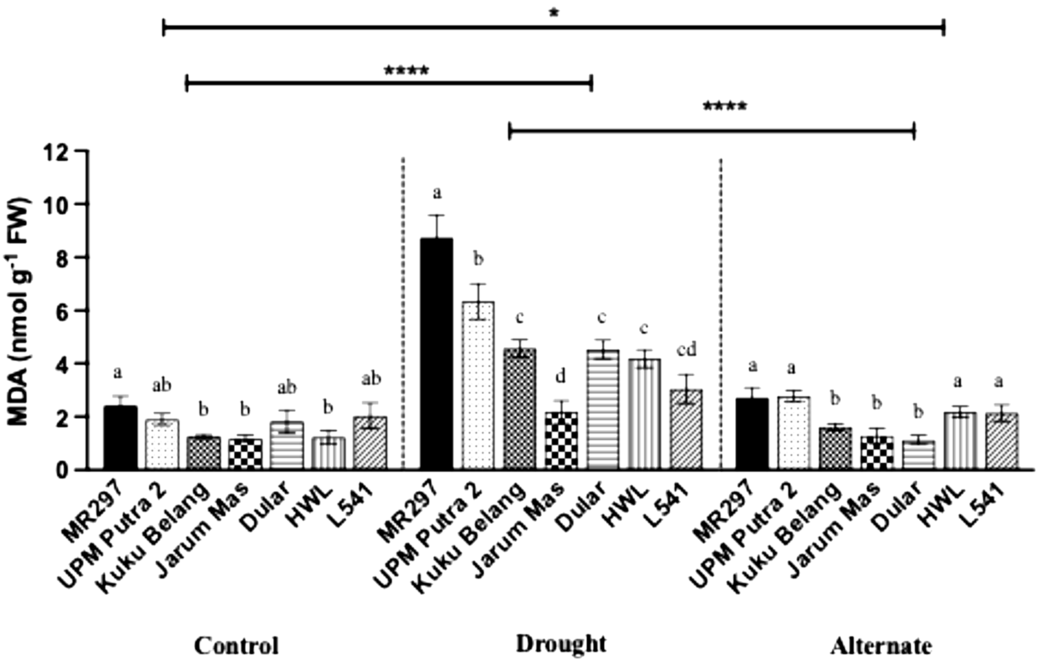

Figure 3 – MDA content in rice seedlings subjected to normal irrigation, drought and alternate drought stress. Data was analysed using one way ANOVA to assess the significance level between means ± SE of n = 5 ($p \leq 0.05$). Comparison was done amongst varieties within the same treatment and values are not sharing common letters differ significantly. Meanwhile, values are not sharing a common superscript (*, **, ***) differ significantly at $p \leq 0.05$, $p \leq 0.001$ and $p \leq 0.001$ (LSD), respectively.

The increase in REL was found to correlate with a significant rise in MDA levels under drought stress conditions. A two-way ANOVA analysis indicated that both continuous and alternate drought treatments significantly influenced REL (Figure 4).

Under control conditions, all plant varieties exhibited REL values ranging from 15% to 26%, indicating minimal membrane damage. In contrast, the imposition of drought conditions significantly elevated REL compared to both control and alternate drought treatments, with HWL and L541 showing the lowest REL values among the treatments. Drought-treated plants exhibited REL values ranging from 48% to 73%, while acclimated plants showed lower REL values between 29% and 44%. Plants exposed to alternate drought condition exhibited REL of less than 50%, a value higher than that observed in the control group. Nonetheless, this impact was comparatively less severe than the effects observed under continuous drought stress. As reported by previous studies [56], REL serves as an indicator of root injury, reflecting the health and functionality of root cell membranes.

There is no definitive standard value for REL; however, typical ranges between 10% to 40%, with values potentially reaching up to 80% were reported in previous studies [56-58]. Nonetheless, plants with acclimated roots to drought stress showed significantly lower root leakage compared to those experiencing acute drought stress exhibiting the importance of root acclimatization in mitigating cellular damage [57]. Both REL and MDA reflect membrane health status, with MDA specifically indicating lipid peroxidation, while REL measuring conductivity due to ion efflux due to damage in cell membranes. In our study, a positive correlation between root REL and MDA levels was observed ($r = 0.3475^{**}$), which is consistent with previous findings that reported a similar relationship in rice subjected to drought stress [59,60]. This correlation portrayed the interconnectedness of lipid peroxidation and membrane integrity under drought conditions, suggesting that both parameters are crucial for assessing plant health and resilience in response to drought.

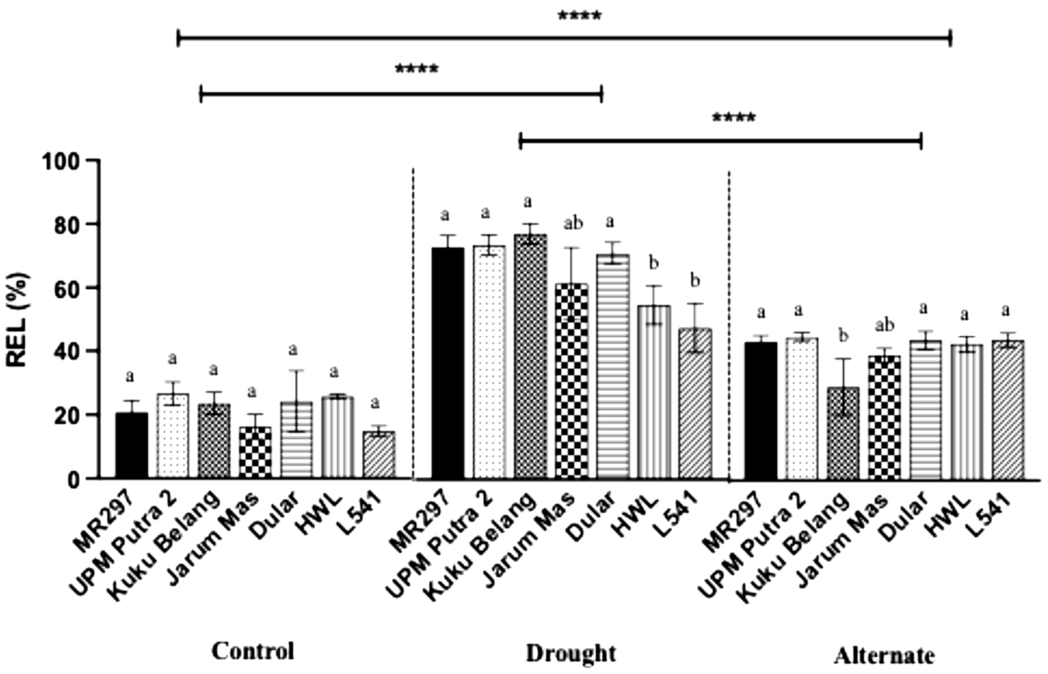

Figure 4 – REL in rice seedlings subjected to normal irrigation, drought and alternate drought stress. Data was analysed using one way ANOVA to assess the significance level between means ± SE of n = 5 ($p \leq 0.05$). Comparison was done amongst varieties within the same treatment and values are not sharing common letters differ significantly. Meanwhile, values are not sharing a common superscript (*, **, ***) differ significantly at $p \leq 0.05$, $p \leq 0.001$ and $p \leq 0.001$ (LSD), respectively.

Conclusion

This study observed that drought conditions led to a decrease in cellular mitotic activity and impaired leaf physiology, as evidenced by reductions in Fv/Fm, total chlorophylls, and total carotenoid content. Additionally, drought induced lipid peroxidation and increased root electrolyte leakage, indicating significant membrane damage. Rice plants subjected to alternating drought treatments, although exhibiting lower values compared to the control, demonstrated enhanced responses across all parameters relative to continuous drought stress. This suggests that acclimation strategies implemented during periods of drought and subsequent rehydration improved the plants' ability to tolerate the typical effects of drought. Furthermore, among the varieties assessed, Kuku Belang and Jarum Mas were identified as possessing superior tolerance traits despite showing an increased root electrolyte leakage.

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Conflict of interest

All authors are aware of the article's content and declare no conflict of interest.

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