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**Original Article**

# Physio-biochemical and anatomical responses of upland rice (*Oryza sativa* L.) genotype during the vegetative stage under drought stress

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## Abstract

Upland rice cultivation in highland regions is often challenged by drought, leading to poor seedling growth and low productivity. This study aimed to investigate the physiological, biochemical, and anatomical responses of the Thai rice landrace Nhonkhab under drought and rewatering conditions. After germination, 28-day-old Nhonkhab rice seedlings were deprived of water for 7 days (35 days old) and then rewatered for 5 days (40 days old). Growth, physiological, biochemical and anatomical characteristics of the 35 and 40-day-old seedlings were evaluated. Results showed that drought stress significantly reduced relative water content (RWC) (-36.38%) but was rapidly restored after rewatering (+12.15%) when compared to the control. After drought stress treatment, malondialdehyde (MDA) content and electrolyte leakage (EL) percentage increased (+150% and +59.37%, respectively), indicating oxidative stress and membrane damage. Growth and developmental adaptations, such as elongated roots and curled leaves, facilitated survival during drought, with leaves unfurling post-rewatering. Following rewatering, rice plants exhibited robust recovery, supported by internal structural adaptations such as increasing stomatal density, thickening of the cuticle layer around the midrib and leaf blades, increasing bulliform cells number and size, and modifications in vascular bundles. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) revealed distinct patterns in growth, physiological, and anatomical traits across control and treatment groups. These findings provide valuable insights for rice production planning in drought-prone highlands and for selecting or breeding drought-resistant cultivars with improved resilience and productivity.

**Keywords**: Electrolyte leakage, Environmental stress, Leaf anatomy, MDA, Upland rice, Water deficiency

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## Introduction

Climate change represents a critical global challenge that is intensifying over time. This will significantly affect the productivity of essential crops and disrupt normal ecosystem functions (Yang et al., 2022). Global warming has significantly influenced the agricultural sector and posed serious challenges to food security (Lynch et al., 2021). Drought, a significant environmental stressor, impacts plant growth and development. It can be chronic in arid regions or unpredictable due to shifting weather patterns during growth periods. The impacts of drought are projected to increase with climate change and producing water scarcity (Alizadeh et al., 2014).

Rice, a major staple food consumed worldwide, is highly sensitive to drought stress, which adversely affects all growth stages by limiting photosynthesis, impairing root and shoot development, and reducing grain yield through increased spikelet sterility and poor grain filling (Hassan et al., 2023). In response, rice plants activate various adaptive mechanisms strategies to survive drought conditions, including avoidance, tolerance, escape, and recovery, which are mediated through morphological, physiological, and biochemical responses (Hassan et al., 2023; Panda et al., 2021). Extensive research has explored its responses across various dimensions, including production (Adjah et al., 2022), morphological and physiological traits (Kumar et al., 2014; Menge et al., 2020; Latif et al., 2023), genomics (Khan, 2012), signaling mechanisms and antioxidative defense systems (Aslam et al., 2022). When facing drought, maintaining favorable leaf water potential after drought supports proper rice flower development, enabling successful pollination and seed formation (Farooq et al., 2024). Relative water content (RWC), a key indicator of tissue metabolic activity, rises during early growth and declines as leaves wilt (Dash et al., 2020; Zhang et al., 2024). Moreover, drought induces stomatal closure by reducing cellular turgor pressure, with ABA hormone, acting as a key signal to guard cells to minimize water loss (Liu et al., 2022). However, this closure also limits carbon dioxide uptake, thereby reducing photosynthesis and overall food production (Yang et al., 2020). In addition, global warming impairs plant stress conditions by altering light reactions in thylakoid membranes under elevated temperatures during drought. This circumstance can damage oxygen-evolving complex proteins, displacing the manganese-containing reaction center and disrupting the association between light-harvesting complexes and photosystem II (Eckardt, 2022). The maximum efficiency of photosystem II, an indicator of its capacity to drive electron transfer using light energy, can be assessed through chlorophyll fluorescence measurements (Wang et al., 2022).

Apart from the effects of drought, restricted cultivation areas represent another critical factor to be addressed. Expanding cultivation areas may not lead to a substantial increase in production, particularly in upland regions where irrigation infrastructure is lacking. Thus, upland rice in highland areas must be cultivated to increase production depending on geographic conditions. Upland rice is a significant crop in intertropical highland areas, providing a crucial source of nutrition for millions of people. It is grown in rainfed, naturally well-drained soil without irrigation, making it a vital crop for areas with limited water resources (Alou et al., 2020). Upland rice cultivation combines mixed agricultural systems, contributing to environmental preservation and soil and water conservation. Sustainable farming approaches are now promoted such as direct planting on permanent plant cover without soil tillage to improve water conservation (Derpsch, 2003). Lack of rain at the start of the growing season frequently impacts the growth of upland rice plants. The seedlings either dry up and die or grow slowly, resulting in low productivity (Ray et al., 2016). Plants have adapted to surviving droughts by altering their life cycle to produce yields earlier (Shanmugavadivel et al., 2019) and regulating leaf water potential by increasing water absorption from the roots to stems and spreading their root systems deeper and wider (Kato et al., 2006). Plants curl and roll their leaves to reduce the surface area exposed to light, which helps lower transpiration. Using this mechanism, plant leaves can better preserve the water content in their tissues. Certain plants exhibit a tolerance to dehydration using these physiological and biochemical response mechanisms to survive drought because plant tissue can tolerate water loss under extreme circumstances. Several biophysical constraints restrict the yield of upland rice including drought (Niang et al., 2018), inadequate soil fertility (Haefele et al., 2014), blast (Sester et al., 2008), weeds (Saito, 2010), and low temperatures (Raboin et al., 2014). Improvement of upland rice variety has mainly focused on research and development of traits including blast resistance, low soil fertility tolerance, and drought tolerance but more research is required.

Upland rice often has low yields and erratic productivity levels due to the influence of dryness at any stage of growth, while in some years the crop fails to produce (Imwichit and Sarutayophat, 2020). Therefore, selecting upland rice varieties that are drought-tolerant at the beginning of the growing season and can survive until yielding is vital to prevent food shortages under drought situations. Phenotype diversity studies of various upland rice varieties have been conducted in 2015 and 2017; twenty-five upland rice genotypes grown under regular irrigation and water restriction were investigated (Lanna et al., 2021). Water deficit in this study reduced grain yield by up to 58%. Key drought tolerance traits included high water use efficiency and strong root development, suggesting that secondary traits should be considered in breeding for better drought tolerance. Moreover, recent findings have increasingly focused on the response of upland rice to drought stress due to its cultivation in rainfed and water-limited environments. da Mata et al. (2023) studied the effects of water deficit during the reproductive stage on upland rice genotypes. Water deficit reduced water potential, gas exchange and grain yield but had no effect on tiller number, shoot biomass or carbohydrate contents. Although the variables distinguished water regime treatments, they did not assist in categorizing the genotypes based on drought tolerance. Expanding knowledge regarding regional types of field rice continues to attract the attention of a limited number of researchers due to yields of lowland rice are generally higher than upland rice systems (Tanaka et al., 2017). As a result, the existing database for upland rice research remains limited. This study contributes to the development of fundamental knowledge in this field. Thus, this study aimed to evaluate the physiological and anatomical responses of the Thai rice landrace Nhonkhab grown under drought and rewatering conditions. This rice variety may exhibit drought tolerance during the seedling stage. While this rice variety has been cultivated in some regions of Thailand, but there is no concrete evidence or formal reports confirming its drought tolerance, particularly during the seedling stage. Therefore, this study is the first report to provide empirical evidence on the drought tolerance performance of this rice variety. The information obtained can be applied to evaluate drought tolerance levels based on anatomical and physiological characteristics in other rice varieties and expand the basic knowledge for upland rice breeding programs in the future.

## Material and Methods

### Experimental design

Nhonkhab rice seeds were germinated in seed trays filled with peat moss under everyday watering. After 14 days, the seedlings were individually transferred into 30 cm diameter pots containing 2 kg of soil and cultured for 14 days in a greenhouse during March and April 2023. The average temperature in the greenhouse during the experiment was 32 ± 2 °C, with a relative humidity of approximately 65 ± 2%. The soil used for cultivation had the following properties: Lime Requirement (LR) = 417 Kg $CaCO_3$/rai, Organic Matter (OM) = 0.27%, Total Nitrogen (N) = 0.012%, Phosphorus (P) = 6.56 mg/Kg, Potassium (K) = 4.73 mg/Kg, Sodium (Na) = 3.28 mg/Kg, and Cation Exchange Capacity (CEC) = 4.90 c mol/Kg. The 28-day-old rice seedlings were then subjected to drought stress by water deprivation for 7 days (35 days old) and then rewatering for 5 days (40 days old) (Figure 1). Four groups were analyzed in this study: drought period control (control-1), drought period treatment, rewatering period control (control-2) and rewatering period treatment. Growth, physiological, and anatomical characteristics of the seedlings were examined at 35 and 40 days for the control and treatment groups. All experiments were conducted at the Department of Biology, Faculty of Science, Khon Kaen University, Thailand.

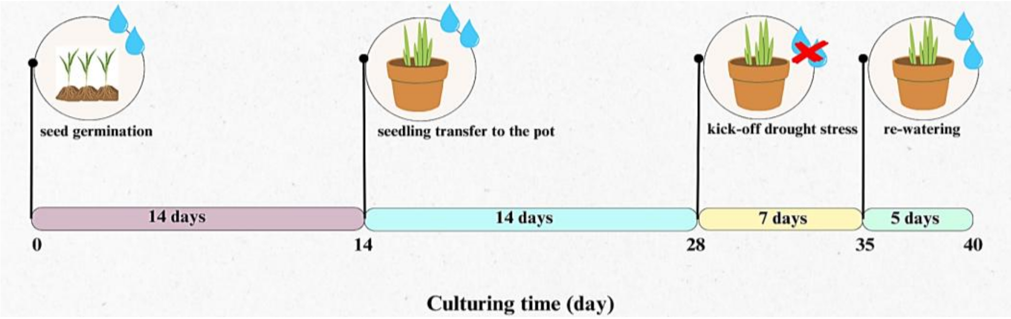

**Figure-1.** Experimental design diagram. Nhonkhab rice seeds were germinated in seed trays for 14 days before being individually transferred into pots and cultured for 14 days prior to drought stress treatment for 7 days (35 days old) and then rewatering for 5 days (40 days old).

## Soil moisture percentage

Soil moisture percentage determination followed the method of Parajuli and Duffy (2013) by first weighing a container with a tightly closed lid for storing soil samples (container weight). Randomly collected soil samples at a depth of approximately 10 cm from the control and treatment groups were placed in the container and weighed to obtain wet soil weight + container weight. The soil was incubated in an oven at 80 °C for 24 h. The weight loss of the soil after drying indicated the weight of water in the soil. Soil moisture percentage by mass was calculated as:

$$\textbf{Soil moisture percent } [\%] = \frac{\textbf{Mw} - \textbf{Md}}{\textbf{Md}} \textbf{ x 100} \qquad \textbf{(1)}$$

where
Mw = wet soil weight (g), and
Md = dry soil weight (g)

## Soil electrical conductivity (ECe)

The soil electrical conductivity measurement followed Kargas et al. (2018). Soil samples were randomly collected before and after the experiment at a depth of approximately 10 cm. The samples were dried and then ground and sieved to remove impurities before weighing and putting in a test tube. Deionized distilled water was added at a ratio of 1:5 as 4 g of soil to 20 ml of deionized water, and the solution was left for 24 h to allow the soil to settle. The electrical conductivity of the soil was measured while it was saturated with water using the PCTestr35 multi-parameter.

## Growth performance

where
V = 80% acetone volume and
W = plant sample weight.

Plant height, tiller number per plant, root length, leaf rolling score, leaf size, leaf number, root fresh weight, root dry weight, shoot fresh weight, shoot dry weight, drought score, and drought recovery score were evaluated after the drought stress treatment for the drought period (35 days old) and the rewatering period (40 days old). Green intensity was evaluated as SPAD units using a Chlorophyll Meter (Konica Minolta SPAD-502 Plus). Change percentages of all parameters between the control and treatment group were calculated as:

$$\textbf{Changes percentage} = [(\textbf{Treatment control}) - \textbf{1}] \textbf{ x 100} \qquad \textbf{(2)}$$

## Chlorophyll content and chlorophyll fluorescence

Chlorophyll content was investigated based on Arnon (1949). Fresh rice leaves (0.03 g) were chopped before transferring into 5 ml of 80% acetone in a test tube and then left in the dark for 48 h. The supernatant from this test tube was used for absorbance measurement by a spectrophotometer at 645 nm (A645) and 663 nm (A663) wavelengths using 80% acetone as the blank. The following equations were used to calculate the amounts of chlorophyll:

$$\textbf{Chlorophyll a (mg/g tissue)} = \frac{(12.7A_{663} - 2.69A_{645}) \text{ x } V}{1000 \text{ x } W} \qquad \textbf{(3)}$$

$$\textbf{Chlorophyll b (mg/g tissue)} = \frac{(22.9A_{645} - 4.68A_{663}) \text{ x } V}{1000 \text{ x } W} \qquad \textbf{(4)}$$

$$\textbf{Total chlorophyll (mg/g tissue)} = \frac{(20.2A_{645} + 8.02A_{663}) \text{ x } V}{1000 \text{ x } W} \qquad \textbf{(5)}$$

Chlorophyll fluorescence was analyzed based on Willits and Peet (2001), while mature leaves were established using a Handy PEA Chlorophyll

Continuous Excitation Fluorimeter for the light-adapted quantum efficiency of PSII (Fv'/Fm') (between 1.00 and 2.00 PM) and the maximum or dark-adapted quantum efficiency of PSII (Fv/Fm) (8.00 PM).

## Relative leaf water content

The relative leaf water content (RWC) was evaluated following Turner (1982). Fresh leaves were cut into 2 cm long pieces, weighed as fresh weight (FW), and then placed in 10 ml of deionized water in a test tube for 4 h. The samples were dried on tissue paper before weighing as saturated weight (SW). The plant materials were then dried using an air oven at 60 °C for 24 h and weighed again as dry weight (DW). The RWC was determined by the formula:

$$\textbf{Relative leaf water content } [\%] = \left(\frac{\textbf{FW-DW}}{\textbf{SW-DW}}\right) \textbf{x100} \quad \textbf{(6)}$$

where
FW = fresh weight (g),
DW = dry weight (g), and
SW = saturated weight (g).

## Malondialdehyde content

Malondialdehyde (MDA) content was investigated based on Sunohara and Matsumoto (2004). Fresh leaves were harvested (1 g) before grinding with 2 ml of 0.1% (w/v) trichloroacetic acid (TCA) and then centrifuging at 14,000 rpm for 5 min at 4 °C. The supernatants were added with 1.5 ml of 0.5% (w/v) thiobarbituric acid (TBA) dissolved in 20% (w/v) TCA. The mixture was boiled for 30 min, and then quickly cooled on ice for 10 min. The absorbance was determined at 532 ($A_{532}$) and 600 nm ($A_{600}$) using 20% (w/v) TCA as the control (blank). The following equation was used to calculate the MDA contents in samples.

$$\textbf{MDA } (\mu\textbf{mol/gFW}) = \frac{(\textbf{A}_{532} - \textbf{A}_{600}) \textbf{ x Vf x Ve}}{\textbf{155 x Va x FW}} \qquad \textbf{(7)}$$

where
$V_f$ = final volume
$V_e$ = trichloroacetic acid (TCA) volume
$V_a$ = solvent volume
FW = plant sample fresh weight.

## Electrolyte leakage (EL)

The EL percentage was evaluated based on Dionisio-Sese and Tobita (1998). The rice leaves were chopped into 1 $cm^2$ pieces. Before measuring the electrical conductivity as $EC_1$, two pieces were placed in a test tube with 10 ml of room temperature deionized water and left in the dark for 24 h. Before performing the second conductivity measurement as $EC_2$ at room temperature, the test tubes were autoclaved for 15 min at 121 °C. Electrolyte leakage percentages were calculated as:

$$\textbf{Electrolyte leakage } (\%) = (\textbf{EC}_1 / \textbf{EC}_2) \textbf{ x 100} \qquad \textbf{(8)}$$

## Anatomical investigation

After treatment, mature leaves were collected, fixed in FAA70 fixative (70% ethyl alcohol, acetic acid, formaldehyde; 90:5:5) for 30 min, and stored in 70% ethyl alcohol. Leaf samples were cut into small pieces and then soaked for 20 min in 15% (v/v) Clorox (sodium hypochlorite) before peeling. A 1% (w/v) Safranin O solution in ethyl alcohol was used to stain the abaxial and adaxial epidermis. The samples were then sequentially soaked in ethyl alcohol, xylene-absolute ethanol, and xylene before mounting using DePeX. The leaf blades were freehand transverse-sectioned and then stained with Safranin O. The stained samples were dehydrated by a continuous series of ethyl alcohol in xylene-absolute ethanol and xylene before mounting using DePeX. Anatomical aspects were investigated using an Olympus CH 30 light compound microscope with 10x objective lens and a Zeiss Primo Star equipped with the MB2004 configuration AxioVision program. Twenty anatomical characteristics were observed based on Taratima et al. (2019).

## Statistical analysis

Data with at least five replications were analyzed using one-way analysis of variance (ANOVA). Duncan's multiple range test was used to examine mean value comparisons with a 95% confidence level. The assumptions of normality and homogeneity of variance were evaluated using the Shapiro-Wilk test and Levene's test at *p*-value = 0.05, respectively. In cases where these assumptions were violated, a logarithmic transformation was applied to the data to enhance conformity with the requirements of parametric statistical analyses. Descriptive statistics were expressed as mean and standard deviation (SD).

Principal component analysis (PCA) was performed to reduce the dimensionality of the data while retaining most of the variance. Components were selected based on the first two largest percentages of explained variance. Hierarchical cluster analysis (HCA) was conducted to express responding patterns of each sample group and to cluster similar observations based on their characteristics. The analysis utilized the average method as the clustering algorithm and the Euclidean distance metric to measure dissimilarity between observations. The data were standardized and scaled before conducting PCA and HCA. All statistical analysis techniques were performed using R version 4.4.1 and RStudio version RStudio 2024.04.2+764 for Windows.

## Results and Discussion

### Growth, physiology and biochemistry investigation

Nhonkhab rice was grown during drought and rewatering periods. Four groups as drought period control (control-1), drought period treatment, rewatering period control (control-2), and rewatering period treatment were investigated. Results showed that growth increased depending on the cultivation period, with average leaf lengths between the groups significantly different. The average fresh weight, dry weight, leaf rolling score, and drought score of the leaves of all experimental groups were significantly different. The rewatering period control (control-2) showed the highest growth followed by the control-1 group (Table 1, Figure 2).

After being subjected to drought stress for 14 days, rice seedling growth decreased in all aspects, except for root length. When there is insufficient water, the pressure inside plant cells decreases. This leads to a reduction in the rate of cell division. The overall biomass therefore decreases. However, rice has adapted to grow longer roots to locate deeper water sources in the soil (Pramesri, 2018). Under water deficiency conditions, root growth takes priority over the shoot growth (Taratima et al., 2023). Rice plants with deep root systems can better survive drought stress (Zhao et al., 2018). Following the rewatering process, the rice stems grew rapidly. This observation aligned with Menge et al. (2020), who reported that rice develops longer roots during drought stress and that these roots become more efficient at absorbing water under rewatering.

**Table-1.** Comparative results of soil measurement, growth, physiological and anatomical characteristics of rice seedlings grown under drought stress before rewatering

| Characteristics | Drought period | | | Rewatering period | | |
|---|---|---|---|---|---|---|
| | Control-1 | Treatment | Change (%) | Control-2 | Treatment | Change (%) |
| Soil temperature | 31.60 ± 0.55 b | 40.86 ± 0.54 a | +29.30 | 32.10 ± 0.36 b | 33.16 ± 1.02 b | +3.30 |
| % soil moisture | 28.60 ± 1.60 a | 4.10 ± 1.11 c | -85.67 | 23.44 ± 1.94 b | 27.20 ± 0.58 ab | +16.04 |
| ECe of soil | 309 .00± 35.85 b | 556.66 ± 67.21 a | +80.15 | 257.66 ± 23.98 b | 331.00 ± 22.33 b | +28.46 |
| Soil pH | 7.20 ± 0.00 a | 7.16 ± 0.03 a | +0.56 | 6.70 ± 0.00 b | 6.70 ± 0.05 b | 0.00 |
| TN (number/clump) | 3.20 ± 0.37 a | 1.80 ± 0.37 b | -43.75 | 4.00 ± 0.44 a | 3.20 ± 0.20 a | -20.00 |
| LN (number/plant) | 3.60 ± 0.24 a | 3.20 ± 0.49 a | -11.11 | 4.00 ± 0.00 a | 3.20 ± 0.20 a | -20.00 |
| SL (cm) | 43.88 ± 2.83 b | 38.12 ± 1.35 b | -13.13 | 57.68 ± 1.64 a | 37.08 ± 2.59 b | -35.71 |
| RL (cm) | 13.38 ± 0.96 b | 15.76 ± 1.27 ab | +17.78 | 19.12 ± 1.60 a | 17.26 ± 1.11 ab | -9.73 |
| LW (cm) | 0.66 ± 0.05 b | 0.60 ± 0.04 b | -9.09 | 0.86 ± 0.05 a | 0.62 ± 0.03 b | -27.91 |
| LL (cm) | 21.08 ± 1.21 b | 16.42 ± 1.90 c | -22.11 | 31.50 ± 1.12 a | 16.72 ± 0.78 c | -46.92 |
| SFW (g) | 3.65 ± 0.24 b | 0.66 ± 0.03 d | -81.92 | 4.71 ± 0.12 a | 1.94 ± 0.35 c | -58.81 |
| SDW (g) | 0.66 ± 0.04 b | 0.23 ± 0.01 d | -65.15 | 0.83 ± 0.02 a | 0.40 ± 0.07 c | -51.81 |
| RFW (g) | 1.05 ± 0.02 b | 0.17 ± 0.00 c | -83.81 | 2.30 ± 0.12 a | 1.00 ± 0.13 b | -56.52 |
| RDW (g) | 0.15 ± 0.01 b | 0.06 ± 0.00 c | -60.00 | 0.25 ± 0.01 a | 0.14 ± 0.02 b | -44.00 |
| Leaf rolling (1-5) | 1.20 ± 0.20 c | 5.00 ± 0.00 a | +316.66 | 2.60 ± 0.24 b | 2.20 ± 0.20 b | -15.39 |
| Drought score of leaf (1-9) | 0.60 ± 0.24 b | 3.80 ± 1.02 a | +533.33 | 0.80 ± 0.20 b | 5.00 ± 0.89 a | +525.00 |
| SPAD unit | 37.45 ± 1.67 a | 26.93 ± 3.97 b | -28.09 | 30.94 ± 0.74 ab | 23.67 ± 2.61 b | -23.00 |
| CHA (mg/g tissue) | 3.31 ± 0.07 b | 4.63 ± 0.02 a | +39.88 | 2.48 ± 0.01 c | 1.78 ± 0.01 d | -28.22 |
| CHB (mg/g tissue) | 1.22 ± 0.02 b | 2.09 ± 0.05 a | +71.31 | 0.68 ± 0.00 c | 0.49 ± 0.00 d | -27.94 |

| | | | | | | |
|---|---|---|---|---|---|---|
| CHT (mg/g tissue) | 4.54 ± 0.08 b | 6.73 ± 0.07 a | +48.24 | 3.16 ± 0.01 c | 2.28 ± 0.01 d | -27.85 |
| DCF | 0.80 ± 0.00 b | 0.77 ± 0.01 c | -3.75 | 0.83 ± 0.00 a | 0.82 ± 0.01 a | -1.20 |
| LCF | 0.75 ± 0.01 ab | 0.72 ± 0.00 bc | -4.00 | 0.77 ± 0.00 a | 0.70 ± 0.02 c | -9.09 |
| RWC (%) | 75.89 ± 2.00 a | 48.28 ± 2.32 b | -36.38 | 67.47 ± 2.75 a | 75.67 ± 5.20 a | +12.15 |
| MDA (µmole/g FW) | 0.06 ± 0.00 c | 0.15 ± 0.00 b | +150.00 | 0.05 ± 0.00 d | 0.16 ± 0.00 a | +220.00 |
| EL (%) | 16.32 ± 3.08 b | 26.01 ± 2.39 a | +59.375 | 13.94 ± 0.80 b | 18.93 ± 0.69 b | +35.80 |
| STD (305.08 x 216.95 µm) | 20.80 ± 1.62 a | 18.20 ± 0.58 ab | -12.50 | 15.20 ± 0.73 b | 20.40 ± 0.98 a | +34.21 |
| STW (µm) | 16.02 ± 0.53 b | 17.15 ± 0.85 ab | +7.05 | 18.46 ± 0.18 a | 18.07 ± 0.74 a | -2.11 |
| STL (µm) | 19.20 ± 0.67 b | 22.84 ± 1.01 a | +18.96 | 21.64 ± 0.48 a | 23.46 ± 0.29 a | +8.41 |
| ECW (µm) | 8.76 ± 0.17 a | 10.72 ± 0.81 a | +22.37 | 11.09 ± 1.05 a | 11.09 ± 0.82 a | 0.00 |
| ECL (µm) | 46.39 ± 2.74 b | 73.31 ± 3.89 a | +58.03 | 79.76 ± 3.54 a | 76.75 ± 3.13 a | -3.77 |
| LT (µm) | 79.70 ± 2.59 a | 69.08 ± 2.34 b | -13.325 | 78.09 ± 3.47 a | 79.22 ± 2.42 a | +1.45 |
| VAM (µm$^2$) | 678.16 ± 37.33 b | 967.84 ± 24.09 a | +42.71 | 974.72 ± 61.82 a | 724.42 ± 33.97 b | -25.68 |
| VAL (µm$^2$) | 521.55 ± 36.79 bc | 570.70 ± 51.82 b | +9.42 | 878.33 ± 37.30 a | 407.78 ± 25.93 c | -53.57 |
| CUM (µm) | 3.11 ± 0.29 c | 5.35 ± 0.20 a | +72.02 | 4.61 ± 0.19 b | 3.60 ± 0.11 c | -21.91 |
| CLM (µm) | 6.58 ± 0.46 b | 7.785 ± 0.29 a | +18.31 | 5.84 ± 0.18 b | 8.35 ± 0.33 a | +42.98 |
| CUL (µm) | 10.46 ± 0.45 a | 10.75 ± 0.37 a | +2.77 | 9.52 ± 0.65 a | 10.52 ± 1.02 a | +10.50 |
| CLL (µm) | 11.92 ± 0.42 ab | 10.52 ± 0.55 b | -11.74 | 9.75 ± 1.00 b | 13.40 ± 1.06 a | +37.43 |
| MVBV (µm) | 87.46 ± 1.52 b | 100.83 ± 3.30 a | +15.27 | 105.80 ± 1.85 a | 90.68 ± 2.69 b | -14.29 |
| MVBH (µm) | 107.96 ± 1.32 b | 120.80 ± 1.34 a | +11.89 | 119.98 ± 3.95 a | 110.60 ± 1.99 b | -7.82 |
| LVBV (µm) | 70.20 ± 4.12 c | 79.83 ± 2.45 b | +13.72 | 91.48 ± 1.08 a | 80.03 ± 3.55 b | -12.52 |
| LVBH (µm) | 93.09 ± 5.88 a | 96.89 ± 1.78 a | +4.08 | 104.03 ± 4.57 a | 93.94 ± 4.15 a | -9.70 |
| SVBV (µm) | 31.68 ± 1.17 a | 26.14 ± 1.77 b | -17.48 | 30.85 ± 1.03 a | 29.30 ± 1.59 ab | -5.02 |
| SVBH (µm) | 27.19 ± 0.98 a | 23.74 ± 1.69 ab | -12.69 | 25.90 ± 1.41 ab | 22.17 ± 0.78 b | -14.40 |
| BCN | 8.80 ± 0.37 a | 8.00 ± 0.31 a | -9.09 | 6.20 ± 0.20 b | 9.00 ± 0.44 a | +45.16 |
| BCVL (µm) | 45.17 ± 2.24 a | 31.25 ± 1.69 b | -30.82 | 42.53 ± 1.40 a | 42.37 ± 3.79 a | -0.38 |
| BCVH (µm) | 93.84 ± 4.97 a | 80.28 ± 5.67 ab | -14.45 | 72.50 ± 1.82 b | 90.99 ± 4.50 a | +25.50 |

The data was expressed as mean and SD; The different letters are the significant difference in columns (*p*-value < 0.05). TN = Tiller number; LN = Leaf number; SL = Shoot length; RL = Root length; LW = Leaf width; LL = Leaf length; SFW = Shoot fresh weight; SDW = Shoot dry weight; RFW = Root fresh weight; RDW = Root dry weight; CHA = Chlorophyll A content; CHB = Chlorophyll B content; CHT = Total chlorophyll content; DCF = Chl fluorescence (dark); LCF = Chl fluorescence (light); RWC = Relative water content; MDA = Malondialdehyde content; EL = Electrolyte leakage; STD = Stomatal density; STW = Stomatal width; STL = Stomatal length; ECW = Epidermal cell width; ECL = Epidermal cell length; LT = Lamina thickness; VAM = Vessel area at midrib; VAL = Vessel area at lamina; CUM = Cuticle thickness at upper side of midrib; CLM = Cuticle thickness at lower side of midrib; CUL = Cuticle thickness at upper side of lamina; CLL = Cuticle thickness at lower side of lamina; MVBV = Size of major vascular bundle at vertical; MVBH = Size of major vascular bundle at horizontal; LVBV = Size of large vascular bundle at vertical; LVBH = Size of large vascular bundle at horizontal; SVBV = Size of small vascular bundle at vertical; SVBH = Size of small vascular bundle at horizontal; BCN = Bulliform cell number; BCVL = Length of bulliform cell at vertical; BCVH = Length of bulliform cell at horizontal

At the start of the growing season, the plants become taller and generate more tillers. When rice is subjected to drought, leaf curling occurs as an important mechanism to prevent water loss by reducing the leaf surface area using interactions between bulliform cells, sclerenchyma, the mesophyll, and vascular bundles. This causes the rice leaves to curl and roll up to reduce the leaf surface area exposed to light and prevent water loss (Latif et al., 2023). Conversely, if the plants can lower the osmotic potential in their leaves, then water can be absorbed by plant roots and slow down the severity of leaf curling. The lack of carbon dioxide gas causes plants to experience dry leaves (Fang and Xiong, 2015). Therefore, the drought stress group that received insufficient water had the highest level of leaf dryness. As a result, the leaf recovery process occurred at a lower level than rice with low leaf dryness scores. This led to a lower degree of leaf recovery compared to rice with low leaf dryness scores (Dien et al., 2017).

The highest leaf green intensity was found in the control-1 group, while the lowest value was observed in the treatment group during the rewatering period. Chlorophyll a, chlorophyll b, and total chlorophyll of the treatment group during the drought period were the highest compared to the other groups. The chlorophyll fluorescence of the control-2 group was highest under dark and light-adapted conditions. Relative leaf water content percentage of the control-1 group was the highest (75.89%), closely followed by the treatment group during the rewatering period (75.67%). The lowest relative leaf water content percentage at 48.28% was found in the treatment group during drought stress.

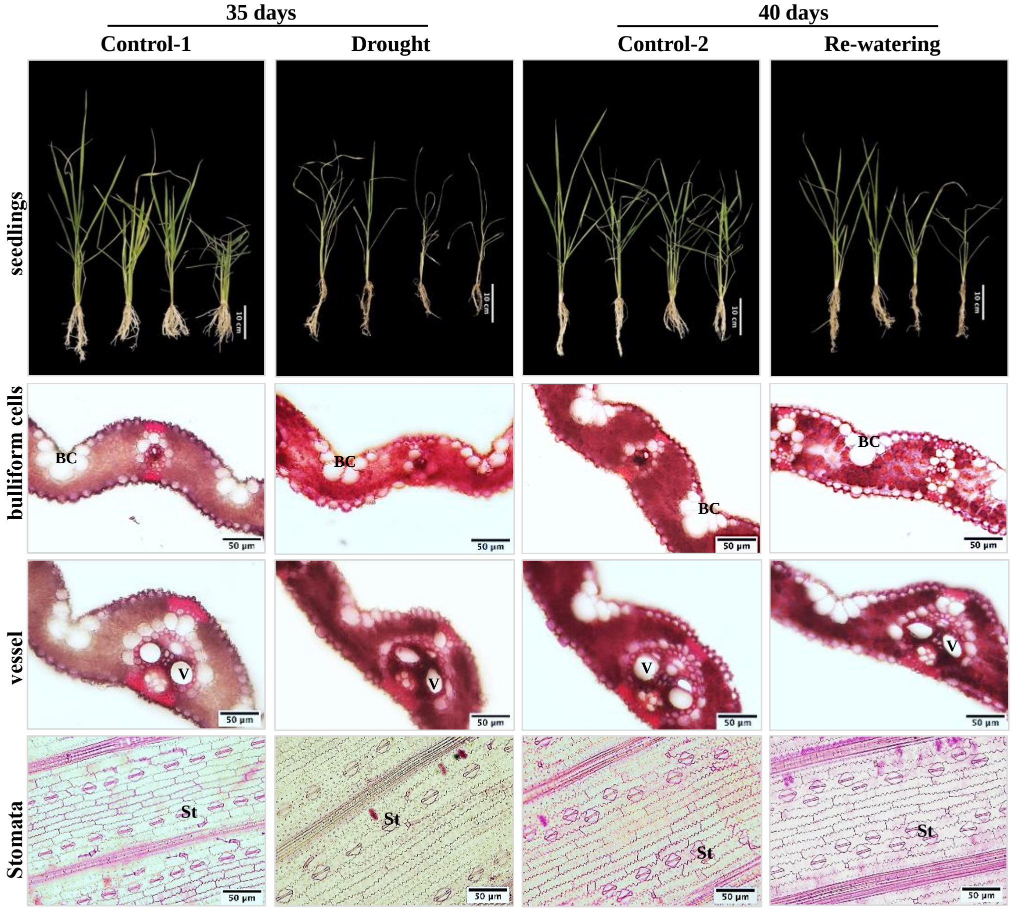

**Figure-2.** Nhonkhab rice seedlings morphology grown under drought (35 days old) and rewatering (40 days old) (Scale = 10 cm). Anatomical features observed from leaf of all treatments presenting bulliform cells (BC), vessel (V) and epidermal cell with stomata (St) (Scale = 50 µm).

The MDA contents of the four experimental groups exhibited significant differences. The highest value was measured during the rewatering period treatment (0.16 µmol/gFM) (Table 1). Electrolyte leakage (EL) percentages of all the experimental groups expressed the same trend as the MDA content. EL of 26.01% was recorded during the treatment of drought stress, significantly different from treatment during the rewatering period (18.93%), the control-1 (16.32%), and the control-2 (13.94%) (Table 1).

During drought, rice has an adaptation mechanism to counteract water deficiency, preventing cellular damage and preserving tissue. Under drought stress, rice in the drought experimental group had the lowest green intensity (SPAD unit) values compared to other groups, while chlorophyll a, chlorophyll b, and total chlorophyll contents were higher than in the control-1 during drought stress. This result is consistent with the findings of Mahatthanaphatcharakun and Taratima (2025), who reported that POKKALI rice maintained high chlorophyll content under drought stress. Moreover, Ahmadikhah and Marufinia (2016) reported that the drought-tolerant rice variety MT58 had the highest accumulation of chlorophyll a, chlorophyll b, and total chlorophyll in its tissues compared to other rice varieties grown under drought conditions. This confirmed that Nhonkhab rice has good drought tolerance characteristics. However, our findings contrast with previous studies, which reported decreased chlorophyll content in sugarcane cultivars F172 and YL6 (Zhang et al., 2015), including certain rice cultivars under drought stress, such as KDML105, MDNK62 (Mahatthanaphatcharakun and Taratima, 2025), and Tubtim Chumphae rice (Kunpratum et al., 2025). However, an increase in chlorophyll content has been documented in some drought-tolerant cultivars, such as POKKALI rice (Mahatthanaphatcharakun and Taratima, 2025). Therefore, the differences and complexities in drought response characteristics among rice varieties require further investigation. Green intensity measures the nitrogen concentration, which is a component in the chlorophyll structure that accumulates in leaves. This method is used to estimate the proportion of chlorophyll that accumulates in plant leaves (Wicharuck et al., 2024). The green intensity of rice seedlings in this study was not correlated with the chlorophyll content because the leaf area of rice leaves is narrow, resulting in less restriction on the sensor's function or possibly an uneven distribution of chlorophyll in the leaves (Ata-Ul-Karim et al., 2013). Shao et al. (2016) studied the structure of chloroplasts using an electron microscope. They found that drought-resistant corn could maintain chloroplasts and thylakoid membranes without swelling, indicating that the chlorophyll structure was not damaged. Plastoglobuli in the thylakoid membrane aid in the production of proteins that respond to and adapt to different conditions, enabling the cell to retain its chlorophyll level (Van Wijk and Kessler, 2017), concurring with the Pearson’s correlation analysis in our experiment.

Rice leaves may develop a thick cuticle layer to allow the accumulation of higher chlorophyll content (Dwiningsih et al., 2021). A positive correlation was found between the chlorophyll content in this study and the thickness of the cuticle layer on the adaxial side of the leaf blade. The chlorophyll content also corresponded to the chlorophyll fluorescence value measured in the dark period (Fv/Fm) and the light period (Fv'/Fm'). In general, plants grown with no interfering factors have chlorophyll fluorescence values between 0.80 and 0.85, showing high photosynthesis efficiency. This characteristic is often used as an index to estimate the potential photosynthetic yield (Oyetunji et al., 2007). Our results showed that the chlorophyll fluorescence value measured during the dark period under drought stress treatment was the lowest compared to the other groups, with an average value of 0.77, which is considered not too low. This result concurred with Faseela et al. (2020) who reported that rice with a dark chlorophyll fluorescence value lower than 0.7 may show deterioration in photosynthesis efficiency and PSII photosystem activity. Some rice varieties grown under drought and heat stress that maintain high total chlorophyll and chlorophyll fluorescence values can still produce high yields after irrigation is resumed (Kumar et al., 2014).

During drought, relative leaf water content was negatively related to MDA content and electrolyte leakage in the drought stress treatment group, while MDA content and electrolyte leakage were positively related. This result concurred with Pamuta et al. (2022), who observed a reduction in the relative water content of rice leaves after a period of dryness. Plants experience stress under drought, which causes them to produce more free radicals within their cells. When free radicals react with fatty acids in the lipid peroxidation process, MDA is obtained. This is one of the final products formed and is commonly used as an index to assess cell stress during such reactions. MDA accumulation in cells causes the destruction of cell membranes and organelle membranes made up of lipids (Melandri et al., 2021), resulting in decreased membrane ability. The passage of substances in and out is therefore reduced and ions and various substances leak out of the cell. This causes cells to lose balance and leads to damage. The rewatering group in this study exhibited high MDA content because rice only partially recovers from drought stress after a long-term lack of water (Dong et al., 2019), consistent with electrolyte leakage that was lower than in the drought stress group.

## Anatomical study

Stomatal density in rice leaves of the control-1 group was highest, and not significantly different from the treatment group grown under the rewatering period. The stomatal size of all experimental groups was not significantly different, except for the control-1 (Table 1). Highest lamina thickness was recorded in the control-1 but was not significantly different from the other groups, except for the drought stress group. Vessel surface area in the midrib and lamina were studied after transverse sectioning. The overall vessel surface area in the midrib was higher than in the lamina. The highest vessel surface areas in the midrib and lamina were observed in the control-2 group at 974.72 ± 61.82 and 878.33 ± 37.30, respectively.

Cuticle and cell wall thickness of the adaxial side of the midrib and lamina was higher during the drought period treatment than in the other groups, with the highest value measured in the treatment group during rewatering. The cuticle and cell wall thickness of the lamina were higher than in the midrib (Table 1). The vascular bundle size of the control (both control-1 and control-2) and treatment groups were significantly different during drought and rewatering conditions. The highest major vascular bundle size was found in the control-2 group, while the highest small vascular bundle size was observed in the control-1 group. The lowest bulliform cell number was counted in the control-2 group, while the highest number was counted in the treatment group during rewatering. Bulliform cell size of the control-1 group was higher than in the other groups (Table 1, Figure 2).

The drought stress group in this study had lower stomatal density, with higher stomatal width and length than the other groups. Our findings contrast with previous reports on other plant species, such as sugarcane cultivars KKU-1999-02 and KKU-1999-03, which showed an increase in stomatal density (Taratima et al., 2021). There is an inverse relationship between stomatal size and density (Hasanuzzaman et al., 2023). Since small stomata are thought to be able to modify stomatal pore area and regulate stomatal conductance more quickly, maximizing water use efficiency over shorter timescales, therefore, a large number of small stomata may also provide more control over stomatal conductance (Raven, 2014; Liu et al., 2017) Some drought-tolerant plants exhibit increased stomatal density under moderate drought stress. This adaptation may enhance the plant's ability to regulate gas exchange and maintain photosynthetic activity under water-limited conditions (Xu and Zhou, 2008). In contrast, severe drought stress often leads to a reduction in stomatal density as a water conservation strategy. This response minimizes water loss through transpiration but may also limit $CO_2$ uptake and photosynthesis (Seleiman et al., 2021). Stomatal density is influenced by environmental cues during leaf development. Drought stress during early growth stages can lead to long-term changes in stomatal patterning, with some genotypes showing greater plasticity than others (Bertolino et al., 2019). Our result exhibited stomatal density decreasing after drought stress treatment, which concurred Esmaeilzadeh-Moridani et al. (2022) who found that field rice adapted to have the lowest stomatal density compared to other groups under drought, related to the activity of the genes that control the appearance of the epidermis. Caine et al. (2023) studied IR64 rice that had been edited with the *EPIDERMAL PATTERNING FACTOR1* (*OsEPF1*) gene from wheat. This gene controls the expression of stomata in number and shape including the size and characteristics of the roots. Rice plants in which this gene was highly expressed had enlarged stomata. During drought stress period, the stomatal density decreased and the roots lengthened significantly during drought exposure. With low biomass, the efficiency of photosystem II remained high until the end of the drought period and displayed stomatal conductance comparable to rice grown under sufficient irrigation. When plants maintain high transpiration and gas exchange rates, they can increase photosynthetic efficiency (Tor-ngern et al., 2015).

Rice leaf thickness under drought stress treatment was thinner than in the other groups due to limited water supply. Under drought, plant cell water is limited, and the genes involved in cell division are reduced. Cell numbers decrease and speed up the plant life cycle (Min et al., 2016). Biswal and Kohli (2013) reported that when rice is subjected to drought, the leaves become thinner due to a reduction in the size and number of mesophyll cells. Khan et al. (2023) studied tobacco leaves. During the dry period, the leaves produced fewer, more dispersed cells in the mesophyll tissue. As a result, the leaf plates became thinner. When rice is impacted by drought, starch in the leaves is broken down, with sugar and carbohydrates accumulating in the leaf sheaths (Pandey and Shukla, 2015). This may be one of the reasons why the rice leaves become thinner.

The thickness of the cuticle and epidermal cell wall in the midrib on the adaxial and abaxial sides of the drought stress treatment group increased more than in the other groups. Generally, plants subjected to water stress exhibit morphological alterations driven by modifications in the plant cell wall. The mechanical regulation of cell expansion by the plant cell wall plays a crucial role in defining cell size and shape (Gall et al., 2015). Thickening of the cuticle and cell wall, especially cell wall elasticity (CWE) helps to maintain turgor pressure or symplast volume within the cells and avoid dehydration when exposed to drought (De Diego et al., 2013; Conesa et al., 2020). It also helps to prevent damage to the mesophyll layer when exposed to high amounts of ultraviolet rays at high temperatures (Salsinha et al., 2021). However, a thick covering of the epidermis was observed under rewatering conditions. Afzal et al. (2017) reported that plants with a thicker epidermis and higher relative water content in their leaves showed drought tolerance.

The xylem area at the midrib of the drought stress treatment group was larger compared to the control-1 group. Upland rice plants have adapted to produce a smaller leaf area and increase the area of the leaf midrib, resulting in an increased ability to control the rate of water transport (Biswal and Kohli, 2013). Lens et al. (2022) reported that plants with large vessel diameters are less likely to form gas bubbles inside the cell than small vessels because small vessels tend to have open ends connected to the external environment. When the stomata are open but there is no water inside the vascular cells/tissue, air bubbles are sucked in. The enlarged gas bubbles interfere with the transport of other substances in the tube, resulting in a pressure reduction within the xylem, leading to the closure of the stomata, reducing the flow efficiency within the tube, and increasing the death rate of plants (Gunnula et al., 2022). Pearson’s correlation analysis results suggested that vessel size in the midrib was positively correlated to rice growth and total chlorophyll content under drought conditions. The results from the PCA graph showed that this characteristic also related to the drought stress treatment group. Therefore, vessels with large areas can survive better under drought conditions. The size of the vascular bundle in the midrib increased and the main vascular bundle in the leaf lamina was not statistically different from the other groups under drought conditions. Small vascular bundle size in the lamina decreased compared to the other groups, with all anatomical characteristics positively related to rice growth.

One mechanism by which rice responds to drought is leaf rolling, which involves the activity of bulliform cells. This study found that the number of bulliform cells in the drought stress treatment group was not significantly different from the control-1 and the rewatering experimental groups. The control-2 group showed the lowest bulliform cell number, consistent with Taratima et al. (2019) who reported that plants with high bulliform cell numbers adapted under drought conditions, while plants with low bulliform cell numbers received enough water. Salsinha et al. (2023) reported that drought-adapted rice had an increased number of small-diameter bulliform cells and tracheids. Bulliform cell size in the drought stress treatment group was the lowest compared to the other groups, consistent with Taratima et al. (2021) who reported that the width of bulliform cells of sugarcane ‘KKU-1999-03’ decreased under drought conditions compared to the control group. Rice cultivars with high leaf rolling scores had significantly decreased bulliform cell size compared to those with medium leaf rolling scores (Gunnula et al., 2022). Therefore, the increased number and smaller size of bulliform cells during drought resulted in rice leaf rolling along the leaf veins to reduce the area for transpiration. After re-irrigation, the leaves increased in number and expanded in size (Zou et al., 2014).

## Principal component analysis (PCA) and Hierarchical cluster analysis (HCA)

After data collection, growth, physiological, and anatomical parameters were collected from rewatering and drought treatments, and their control groups. Principal component analysis (PCA) was performed with the same dataset to explain the effects of various parameters on control and treatment groups and reveal the relation among the recorded parameters. The PCA results were displayed as scree, score and loading plots (Figure 3A-C). Scree plot exhibited 10 separate components, of which percentages of explained variance varied between 32.27 to 2.27% (Figure 3A). Score and loading plot were created from the components providing the largest explained variance, principal components (PC) 1 and 2. PC1 and PC2 covered 32.3% and 21.4% of the variance, respectively providing 53.7% cumulative variance. The score plot shows that all data from different treatments were clustered into four clusters. The control group of rewatering was clustered at the third quadrant, while the control group of drought treatment were clustered with rewatering treatment. Another group that was separated from others was the treatment of drought stress, which was clustered at the fourth quadrant (Figure 3B).

The influence of collected parameters on the clustering was exhibited by the loading plot (Figure 3C). The plot showed that CHA, CHB, CHT, and EL were positively correlated to each other and these parameters resulted in the clustering of drought treatment in the score plot. These parameters also showed strongly negative correlations with other parameters especially growth parameters like shoot and root biomass, leaf number, tillering number, leaf size. These growth parameters also affected the cluster of control group of rewatering in the score plot. The groups of control of drought and rewatering treatments were clustered based on the effects of cuticle-cell wall thickness, MDA content, and bulliform cells that negatively correlated with growth parameters. The responding patterns on growth, physiological, and anatomical parameters of those treatments on drought and rewatering treatments were clarified with hierarchical cluster analysis.

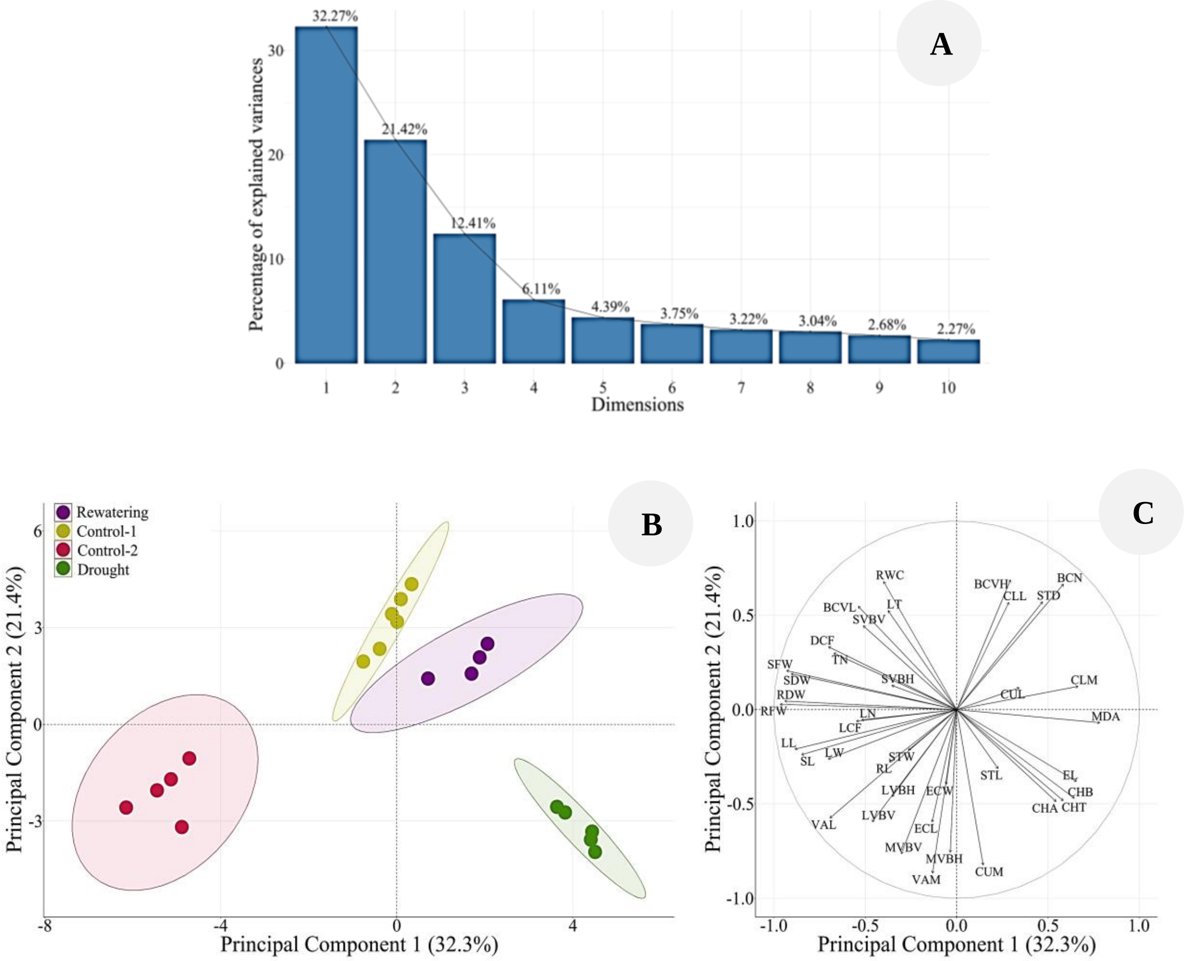

**Figure-3.** Principal component analysis of Nhonkhab rice grown under drought and rewatering conditions. (A) scree plot; (B) score plot; (C) loading plot.

TN = Tiller number; LN = Leaf number; SL = Shoot length; RL = Root length; LW = Leaf width; LL = Leaf length; SFW = Shoot fresh weight; SDW = Shoot dry weight; RFW = Root fresh weight; RDW = Root dry weight; CHA = Chlorophyll A content; CHB = Chlorophyll B content; CHT = Total chlorophyll content; DCF = Chl fluorescence (dark); LCF = Chl fluorescence (light); RWC = Relative water content; MDA = Malondialdehyde content; EL = Electrolyte leakage; STD = Stomatal density; STW = Stomatal width; STL = Stomatal length; ECW = Epidermal cell width; ECL = Epidermal cell length; LT = Lamina thickness; VAM = Vessel area at midrib; VAL = Vessel area at lamina; CUM = Cuticle thickness at upper side of midrib; CLM = Cuticle thickness at lower side of midrib; CUL = Cuticle thickness at upper side of lamina; CLL = Cuticle thickness at lower side of lamina; MVBV = Size of major vascular bundle at vertical; MVBH = Size of major vascular bundle at horizontal; LVBV = Size of large vascular bundle at vertical; LVBH = Size of large vascular bundle at horizontal; SVBV = Size of small vascular bundle at vertical; SVBH = Size of small vascular bundle at horizontal; BCN = Bulliform cell number; BCVL = Length of bulliform cell at vertical; BCVH = Length of bulliform cell at horizontal.

The same dataset from PCA was analyzed with hierarchical cluster analysis (HCA) and visualized as a heat map to illustrate Nhonkhab rice's responses to drought and rewatering treatments (Figure 4). The heat map showed consistent results with the PCA results. Nhonkhab rice from different treatment groups were classified into four groups based on distinct response patterns across parameters: (1) drought treatment, (2) rewatering treatment, (3) drought control, and (4) rewatering control. In addition, the measured parameters were clustered into four groups, reflecting trends of alteration in Nhonkhab rice under varying growth conditions. Group 1, containing MDA, cuticle-cell wall thickness, stomatal density, and bulliform cell number and size, was moderately to highly expressed in drought, rewatering and drought control. Group 2 included chlorophyll content and EL, showing high expression under drought treatment, while Group 3 comprised mostly growth parameters, chlorophyll fluorescence, and some anatomical characteristics, which were highly expressed in the rewatering control group. The final group included the remaining anatomical parameters, which were also expressed in the rewatering control group but showed no definitive expression pattern in other groups. PCA and HCA results clearly demonstrated the clustering of Nhonkhab rice under drought and rewatering conditions, revealing distinct growth, physiological, and anatomical expressions in response to varying cultivation conditions.

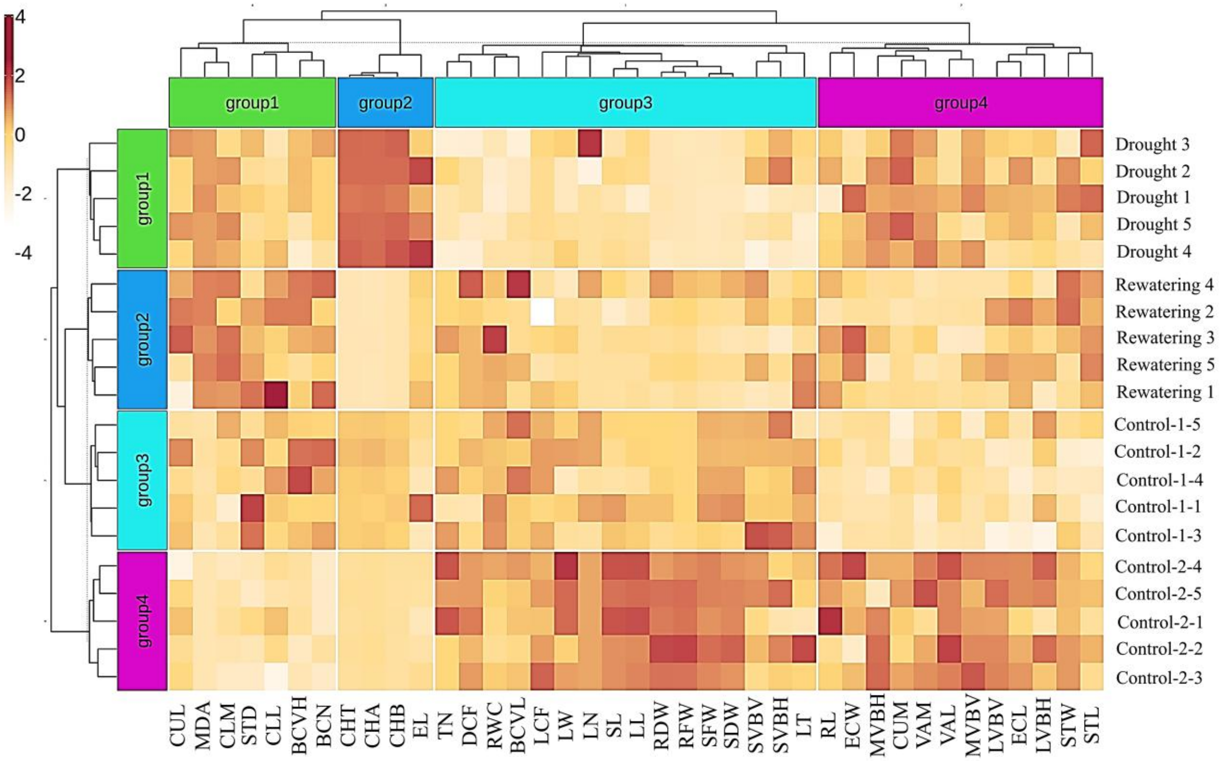

**Figure-4.** Hierarchical cluster analysis (HCA) results and a heat map showing the responding patterns of control groups, drought, and rewatering treatments on four groups of parameters**.**

TN = Tiller number; LN = Leaf number; SL = Shoot length; RL = Root length; LW = Leaf width; LL = Leaf length; SFW = Shoot fresh weight; SDW = Shoot dry weight; RFW = Root fresh weight; RDW = Root dry weight; CHA = Chlorophyll A content; CHB = Chlorophyll B content; CHT = Total chlorophyll content; DCF = Chl fluorescence (dark); LCF = Chl fluorescence (light); RWC = Relative water content; MDA = Malondialdehyde content; EL = Electrolyte leakage; STD = Stomatal density; STW = Stomatal width; STL = Stomatal length; ECW = Epidermal cell width; ECL = Epidermal cell length; LT = Lamina thickness; VAM = Vessel area at midrib; VAL = Vessel area at lamina; CUM = Cuticle thickness at upper side of midrib; CLM = Cuticle thickness at lower side of midrib; CUL = Cuticle thickness at upper side of lamina; CLL = Cuticle thickness at lower side of lamina; MVBV = Size of major vascular bundle at vertical; MVBH = Size of major vascular bundle at horizontal; LVBV = Size of large vascular bundle at vertical; LVBH = Size of large vascular bundle at horizontal; SVBV = Size of small vascular bundle at vertical; SVBH = Size of small vascular bundle at horizontal; BCN = Bulliform cell number; BCVL = Length of bulliform cell at vertical; BCVH = Length of bulliform cell at horizontal.

PCA was employed with Nhonkhab rice to demonstrate the overview and data clustering because of drought and rewatering. The score plot of PCA revealed four clusters of Nhonkhab rice, while the loading plot exhibited the effects of different parameters on the clusters and showed relations among them. Analysis of the loading plot revealed a strong positive correlation among all growth parameters, including biomass, size, and the number of vegetative parts, which contributed to the clustering of the control-2 group in the score plot. Additionally, these parameters also positively correlated with chlorophyll fluorescence and relative water contents. Consistence in chlorophyll fluorescence and relative water contents throughout the experiment period of the control rewatering group reflect the stability of photosynthetic rate, water homeostasis, and metabolic activities inside the plant cells (Jarin et al., 2024). The favorable growth of Nhonkhab rice in the control rewatering group was further supported by the lower levels of electrolyte leakage and malondialdehyde (MDA), which serve as critical indicators of cellular injury and oxidative stress (Melandri et al., 2021). In addition to physiological and growth parameters, the larger vascular bundles and stomata compared to other groups supported the clustering of control-2 (Figure 3B).

Compared to control-2, the drought group was clustered with higher chlorophyll contents, and electrolyte leakage, which positively correlated with MDA content (Figure 3A). Usually, the elevated levels of electrolyte leakage and MDA content could be observed in plants exposed to abiotic stress, like *Manihot esculenta* and *Portulaca oleracea* that were treated with cold and salt stress, respectively (Hnilickova et al., 2021). Drought stress induces severe reduction of water content and water use efficiency (WUE) leading to disruption of photosynthetic rates, malfunction of metabolic activities, and membrane impairment (Hassan et al., 2023). The negative correlation between relative water content (RWC) and stress indicators, e.g. electrolyte leakage and MDA, as expressed in the loading plot also proved the negative results of drought stress on Nhonkhab rice (Figure 3C). Drought stress significantly impacts chlorophyll content in rice seedlings, often leading to a reduction due to photo-oxidative damage by increases reactive oxygen species (ROS) production which can degrade chlorophyll molecules and impair photosynthetic machinery (Valladares and Pearcy, 1997; Vijayaraghavareddy et al., 2022). ROS are produced from different cell compartments to act as molecular signaling to cope with stress. However, prolonged or high-level drought stress induces closure of stomata and leakage of electron from photosystem I to oxygen and lead to overproduction of ROS (Hassan et al., 2023; Jarin et al., 2024). Excessive ROS can impair membrane by lipid peroxidation and generate MDA, and disrupt cell compartments, like mitochondria and chloroplasts. These reactions result in increasing MDA content and declining chlorophyll content, which were evident in several apple cultivars (Mihaljevic et al., 2021). Moreover, some abiotic stress such as salinity stress or drought stress upregulates enzymes like chlorophyllase, which break down chlorophyll, further reducing chlorophyll content (Sherin et al., 2022). Studies have shown that drought-tolerant rice varieties often maintain higher chlorophyll content compared to susceptible varieties, as they possess better antioxidant systems to mitigate ROS damage and maintain photosynthetic efficiency (Hasanuzzaman et al., 2013). The elevated chlorophyll contents during drought treatments were also reported in *Lotus corniculatus* (González-Espíndola et al., 2024), *Vitis* hybrids (Rustioni and Bianchi, 2021), and *Triticum aestivum* (Nikolaeva et al., 2010). Besides, MDA content did not contribute only to the drought treatment group, but increased MDA content also was observed in the rewatering treatment accompanied with the leaf cuticle thickness, number, and size of bulliform cells that were increased in both drought and rewatering treatments (Figure 3C). Therefore, the PCA results revealed distinct clustering patterns among all treatments and elucidated the specific parameters that contributed critically to these observed groupings.

To highlight the dynamic changes of measured parameters across all treatments, HCA was performed and visualized as a heat map using a similar dataset with PCA. The heat map revealed the consistent cluster with the score plot of PCA. Considering each group of parameters, growth parameters and relative water contents belonging to group 3 exhibited high expression in the control-2 group (Figure 4). Besides, the chlorophyll fluorescence (dark and light), included in group 3, also expressed similar levels. The results indicated that high relative water content resulted in high chlorophyll fluorescence, and both parameters can be used to predict drought tolerance efficiency of plants (Reis et al., 2021). There were not only the growth and physiological parameters that were prominently expressed in control-2 treatments, but several anatomical parameters were also incorporated within groups 3 and 4, demonstrating enhanced expression under control-2 treatment conditions. The vascular bundle and epidermal cell size of Nhonkhab rice subjected to control-2 group was larger than other groups as evidenced by the heightened intensity observed in the heatmap (Figure 4). The larger vascular bundle and xylem in well-watered plants, such as *Triticum aestivum* (David et al., 2017) and *Cyamopsis tetragonoloba* (Narayan et al., 2023), because enlarged xylem and vascular area contribute to increased leaf hydraulic conductivity, thereby facilitating more efficient water and nutrient transport, which consequently promotes growth performance (Horike et al., 2023), as observed in the treatment of control-2.

In addition to vascular bundle size, cuticle thickness has been considered as a biomarker for plants exposed to drought stress. Cuticle is the waxy layer covering the epidermis of plant leaves and acts as the barrier that restricts water loss from leaves (Chen et al., 2020). The increased cuticle thickness in plant leaves that are subjected to drought stress is observed in several plant species, like *Triticum aestivum* (Bi et al., 2017), *Camellia sinensis* (Chen et al., 2020), and also Nhonkhab rice in this study. Cuticle thickness parameters were grouped into group 1 and they were declined in the treatments of control-1 and control 2 (Figure. 4). However, Nhonkhab rice under drought stress conditions exhibited cuticle thickening, which coincided with elevated electrolyte leakage and MDA accumulation. Cutinization and cell wall thicker has been considered as anatomical adaptation of plants to maintain water content inside the plant cells (Jarin et al., 2024). The upregulation of genes related with lignin biosynthesis, such as *PuC3H35* in *Populus ussuriensis* and the activation of enzymes, like phenylalanine ammonia lyase (PAL) and cinnamate 4-hydroxylase (C4H) in *Medicago sativa* were induced by drought conditions (Yang et al., 2023). Water deficit conditions also result in the accumulation of plant cell wall constituents like cellulose, hemicellulose, and pectin (Gall et al., 2015). These studies indicated that cuticle and cell wall thickness is directly influenced by drought as noticed in our study.

Stomatal density in group 1 represents another feature subject to significant modification when plants are exposed to drought conditions. The higher stomatal density in drought treatments compared to control-1 and control-2 negatively correlated with stomatal size, e.g. high stomatal density always accompanied with small stomatal size (Yavas et al., 2024). Reduced stomatal size is plant anatomical response to cope with water-deficit condition, because smaller stomata could provide faster stomatal closure to maintain relative water content in plant leaves (Driesen et al., 2023). Plants with smaller stomata could compensate for lower stomatal conductance and photosynthesis by increasing stomatal density (Buckley et al., 2020). In this study, increased stomatal density and smaller stomatal size were noticed in drought stress, and *vice versa* in control-2 (Figure 4). The results proved the inverse relation between stomatal density and size of Nhonkhab rice in drought stress response. Besides, decreasing vascular bundle size and relative water content were noticed when the rice was treated with drought stress, which indicated that drought stress induced leaf anatomical changes in Nhonkhab rice to response to water deficit conditions.

Chlorophyll contents, which were clustered in the same group with electrolyte leakage (group 2), highly expressed after Nhonkhab rice was treated with drought treatments. Interestingly, chlorophyll contents were elevated in drought treatment when compared to control-1 and control-2. The phenomenon was explained that in the early period or moderate levels of drought stress, plants upregulated enzymes related to chlorophyll biosynthesis to maintain photosynthetic rates. In peanuts, *AhGLK1* gene, regulating chloroplast development and light harvesting complex functions, was upregulated during drought stress and facilitated drought tolerance in *ahglk1 Arabidopsis* (Liu et al., 2018). Although early drought periods, approximately 10-15 days, induced chlorophyll accumulation in *Capsicum frutescens*, the prolonged stress resulted in drastically decreasing chlorophyll contents (Yusuf and Hamed, 2021). In our study, the parameters in group 2 recovered to the equivalent levels with control groups after rewatering, which indicated that the membrane integrity and chlorophyll contents of Nhonkhab rice could be recovered when the rice was rewatered.

After rewatering, growth parameters and vascular bundle size, belonging to group 3, demonstrated a slight recovery, approaching levels comparable to those observed in the Control-1 group. In contrast, significant recovery was noted in the parameters of group 2, including chlorophyll content and electrolyte leakage, as their values decreased to levels consistent with those of the other treatment groups. The observed reduction in group 2 parameters following rewatering suggests that Nhonkhab rice exhibits physiological recovery capacity when water availability is restored. Despite the observed recovery in growth, physiological, and certain anatomical characteristics, cuticle thickness and stomatal density showed no recovery under the rewatering treatment. The results indicated a limited capacity for recovery in these traits, though Nhonkhab rice was rewatered (Figure 4). Our results clearly demonstrated that Nhonkhab rice could adapt to drought stress through chlorophyll accumulation and increase in cuticle and cell wall thickness. Although their electrolyte leakage and chlorophyll contents could be recovered into the control levels after rewatering period, Nhonkhab rice's growth, relative water content, MDA and certain anatomical parameters did not fully recover, potentially leading to eventual plant death. This study indicates the severe effects of drought stress accompanying global warming and heat stress, which threaten landrace rice and diversity of rice. The understanding of drought stress responses in rice from this study can be used as key traits in combination with related molecular markers to breed drought-tolerant rice varieties efficiently. However, appropriate crossbreeding programs or biotechnological techniques should be preferred for rice improvement prior to field testing and evaluation of the success. Incorporating drought-tolerant traits into breeding pipelines through a combination of traditional breeding, molecular marker, and biotechnological tools should be taken into consideration based on adequate information to develop high-throughput phenotyping methods to efficiently screen large populations for drought tolerance traits. Therefore, findings from this study can be applied for rice breeding and to evaluate drought tolerance in other rice landraces.

## Conclusion

This study elucidated the physiological and anatomical adaptations of Nhonkhab rice under drought stress. The rice plants exhibited key drought-responsive traits, including an extended root system and reversible leaf curling, which facilitated survival under limited water availability. Structural modifications, such as increased cuticle thickness and adjustments in stomatal density, were observed alongside anatomical changes in vascular bundles and bulliform cells. Elevated levels of MDA and electrolyte leakage indicated oxidative stress responses, while the maintenance of chlorophyll content and photosynthetic efficiency reflected the plant's sustained metabolic activity during stress. These adaptive traits are likely the result of long-term selection under rainfed conditions. Overall, the findings underscore the high drought resilience of Nhonkhab rice and its potential utility in breeding programs aimed at improving drought tolerance. Nonetheless, further research is required to assess its yield performance at the panicle stage and to investigate its responses under combined stresses such as salinity and heat.

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## Contribution of Authors

Sutthachai S: Methodology, investigation, data curation and writing original draft.
Trunjaruen A: Software, formal analysis, writing original draft, review and editing.
Mahatthanaphatcharakun P: Investigation and data curation.
Taratima W: Conceptualization, funding acquisition, supervision, writing original draft, review and editing.

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