Kunpratum N *et al.* (2025)
Notulae Botanicae Horti Agrobotanici Cluj-Napoca
Volume 53, Issue 1, Article number 14060
DOI:10.15835/nbha53114060
Research Article

Physiological and anatomical adaptations of rice (*Oryza sativa* L.) grown under drought stress

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Abstract

Drought stress can affect significant productivity and quality attributes in rice. This research assessed the impact of drought stress on the physiological and anatomical adaptations of 'Tubtim Chumphae' rice. Seedlings were cultivated for 45 days in soil before being subjected to drought stress. The seedlings were divided into two groups as full water capacity treatment and drought stress treatment for 21 days before rewatering for 10 days. Dehydration from drought stress reduced rice seedling plant height, tiller number, leaf size, and fresh and dry weight while leaf rolling score increased. The recovery process from drought stress impacted the physiological characteristics. Relative water content and chlorophyll fluorescence decreased while green intensity (SPAD value), chlorophyll content, electrolyte leakage percentage, and malondialdehyde (MDA) content increased. Anatomical studies using free-hand section and peeling techniques revealed that water deficit reduced vascular bundle size, bulliform cell size, stomatal size, and epidermal cell (short cell) size while leaf thickness, cuticle and cell wall thickness and bulliform cell number increased. Our results provide useful information on rice seedling adaptation and response to drought for use in further studies of 'Tubtim Chumphae' rice and other cultivars.

Keywords: anatomical adaptation; drought stress; physiological characteristics; water deficit

Introduction

Global warming has resulted in climate change with droughts in many areas impacting the growth, quantity, and quality of crops (Wang *et al.*, 2003; Rollins *et al.*, 2013). Drought results in yield losses of up to 60%, depending on the type of plant, the stage of development, and the severity and duration of the dry spell (Jedmowski *et al.*, 2015). The amount of water or moisture in the soil is insufficient, leading to drought stress. Water makes up to 80-95% of cell biomass and lack of water results in changes in developmental characteristics

Received: 10 Aug 2024. Received in revised form: 30 Dec 2024. Accepted: 20 Jan 2025. Published online: 20 Feb 2025.
From Volume 49, Issue 1, 2021, Notulae Botanicae Horti Agrobotanici Cluj-Napoca journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

including physiology, biochemistry, and molecular anatomy in plants (Salehi-Lisar and Bakhshayeshan-Agdam, 2016; Aslam *et al*., 2022). These changes vary depending on diverse factors. For example, plants curl their leaves to minimize exposed evaporation surface area and reduce leaf transpiration when they do not get enough water. Moreover, closing of the leaf stomata as a result of drought stress leads to changes in water content, chlorophyll content, and cell membrane structure which affect the photosynthetic rate (Li *et al*., 2023; Asargew *et al*., 2024).

Plants are unable to tolerate severe drought conditions. Persistent droughts have increased the occurrence of significant outbreaks of rice diseases, such as caused by brown planthopper (Liao *et al*., 2023). This aphid sucks the sap from rice plants during hot, humid weather. Infected rice plants exhibit curled, dry and burned leaves and major outbreaks lead to plant death (Liang *et al*., 2022). Under persistent drought stress, plants generate large amounts of reactive oxygen species (ROS). These are toxic and accumulate inside the cells, leading to lipid peroxidation, the breakdown of proteins in the cell membranes, and destruction of the genetic material within the cells (Nadarajah, 2020). The ability of plants to absorb water under drought situations through their roots decreases due to the increase in osmotic pressure and disruption of the cell membranes, contributing to electrolyte leakage (Boaretto *et al*., 2014).

Compared to other crops, rice requires large amounts of water during its life cycle, especially in germination and the early stage of seedling growth (Panda *et al*., 2021). Drought stress severely affects rice production even for short periods (Pandey and Shukla, 2015; Hussain *et al*., 2022). Some drought-tolerant rice varieties have a high recovery index after drought condition, enabling them to produce a higher complete seed yield compared to varieties with low recovery ability, as detected by physical, physiological and biochemical measurements (Sandeep and Godi, 2023). Previous reports proved that some rice varieties such as MT58 showed drought-tolerant characteristics as accumulation of chlorophyll content in its cells and tissues (Ahmadikhah and Marufinia, 2016). Moreover, the 'Pokkali' rice exhibited a well-developed root system that can effectively absorb water and nutrients from the soil under drought conditions (Jacob and Subramannian, 2022). The effect of drought on rice growth requires detailed investigation to develop rice cultivars that are resistant to arid conditions.

Thailand frequently encounters drought challenges and rice is one of the key economic crops. Most rice growing areas in Thailand still rely primarily on rainwater or irrigation. This research studied the physiological and anatomical responses of 'Tubtim Chumphae' rice under drought stress during the seedling stage. 'Tubtim Chumphae' rice seeds have a red seed coat that is gaining popularity in Thailand. This variety has excellent nutrition, antioxidant properties and high phenolic and flavonoid contents. It is also rich in numerous vitamins that lower cholesterol levels in the bloodstream and reduce the risk of cancer while the low amylose content is acceptable for people with diabetes (Srinang *et al*., 2023). 'Tubtim Chumphae' is now one of the main rice varieties that generates good income for farmers. 'Tubtim Chumphae' rice growth has been studied under abiotic salinity stress of seedlings (Taratima *et al*., 2023a) and drought stress of callus (Taratima *et al*., 2023b) but no research has addressed the physiological, biochemical, or anatomical responses of seedlings to drought stress. This research offers crucial foundational knowledge for understanding the effects of drought stress on the growth, physiology, and anatomy of the seedling stage of 'Tubtim Chumphae' rice, with benefits for further research in this and other rice varieties.

Materials and Methods

Plant materials and drought stress treatment

Healthy seeds of 'Tubtim Chumphae' rice were soaked in distilled water for 24 h before planting on wet tissue paper for 2 days. Germinated seeds were transplanted into seed trays containing peat moss with everyday watering. Fourteen-day-old seedlings were transferred into pots (2 seedlings/pot) containing 1.9 kg soil (17 cm

pot diameter) with 0.62 g of fertilizer (18-22-0 of N:P:K) per pot. The rice seedlings were cultured for 24 days under greenhouse at the Department of Biology, Faculty of Science, Khon Kaen University, Thailand during June and July 2022 with average temperature 29.5 °C, average minimum temperature 24.5 °C, average maximum temperature of 33.5 °C, average relative humidity 73.5%, and average rainfall 137.3 mm. Drought stress was initiated in 24-day-old rice seedlings by withholding water for 21 days (until they were 45 days old), followed by rewatering for 10 days (until they were 55 days old). Data including soil features, plant growth, and physiological and anatomical characteristics were recorded at 45 days and 55 days for both the control and treatment groups.

Soil moisture content (SMC) and electrical conductivity (ECe)

Soil samples were randomly collected from the control and treatment pots at 0-5 cm depth and weighed before heating in a hot air oven at 80 °C for 24 h and then weighed again. The weight decrease after drying was the weight of water in the soil. Soil moisture percentage was calculated using the following equation.

$$\text{Soil moisture content } [\%] = \frac{\text{Mw} - \text{Md}}{\text{Md}} \times 100$$

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

The electrical conductivity (ECe) was measured according to Kargas *et al*. (2018). Soil samples were randomly collected from the control and treated pots at 0-10 cm depth, air-dried, ground, and sifted with a sieve. The dried sifted soil was mixed with deionized water at a ratio of soil to water 1:5 (3 g soil: 15 ml deionized water) in a test tube and kept at room temperature for 24 h to allow the soil to settle. The supernatant was then subjected to electrical conductivity measurement using a PL700 Series Bench Top Meter.

Plant growth

Plant height, root length, tiller number per clump, leaf number per clump, leaf size, shoot fresh weight, shoot dry weight, root fresh weight and root dry weight were recorded after 21 days of drought stress treatment (45 days old) and rewatering for 10 days (55 days old). Leaf rolling scores (RS) at 1 to 5 followed O'Toole and Moya (1978) as 1 (no leaf curling), 2 (leaf edges slightly curved), 3 (leaf edges curve closer together as semicircular), 4 (leaf margins gradually converge to form a single edge), and 5 (leaves have curving edges that eventually wrap into one).

Drought scores

Leaf drought scores (0-9) were evaluated according to IRRI (2002). The degree of leaf dryness of the control group and drought stress group were assessed at 6 levels as 0 (no symptoms), 1 (slightly dry at leaf apex), 3 (dry leaf apex extends to 25% of the total leaf area), 5 (more than 25% to 50% of leaf apex dry), 7 (more than 70% of the leaf apex are dry), and 9 (whole rice leaves dry and dead).

Drought recovery score (DRS)

The recovery levels (1-9) from drought stress of rice plants after rewatering for 10 days were evaluated according to IRRI (2002), divided into 5 levels as 1 (90-100% able to recover and create new leaves or shoots), 3 (70-89% able to recover and create new leaves or shoots), 5 (40-69% able to recover and create new leaves or shoots), 7 (20-39% able to recover and create new leaves or shoots), and 9 (0-19% able to recover and create new leaves or shoots).

Green intensity, chlorophyll content and chlorophyll fluorescence

Leaf green intensity was measured between 12.00 h and 14.00 h using a Chlorophyll Meter SPAD - 502 Plus, with chlorophyll content determined according to Arnon (1949). Fresh leaves (30 mg) were chopped and

transferred into a test tube containing 5 ml of 80% acetone and kept in the dark for 48 h. The supernatant absorbance was measured by a spectrophotometer at 645 nm (A645) and 663 nm (A663) wavelengths with 80% acetone as the blank. Chlorophyll contents of all investigations were calculated using the following equations:

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

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

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

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

Chlorophyll fluorescence measurement was conducted according to Willits and Peet (2001) using a Handy PEA Continuous Excitation Chlorophyll Fluorimeter for both light-adapted quantum efficiency of PSII (Fv'/Fm') and dark-adapted quantum efficiency of PSII (Fv/Fm).

Relative water content

The relative water content (RWC) was tested according to Turner (1981). Fresh leaves were cut into 2 cm long pieces and then immediately weighed (fresh weight) before transfer into a Petri dish containing 10 ml of deionized water for 4 h to allow the leaves to fully absorb water. The leaves were then placed on tissue paper to dry before weighing to determine the weight of plant leaves fully saturated with water (saturated weight; SW). Plant samples were then dried in a hot air oven at 60 °C for 24 h before weighing to determine the dry weight (DW). The relative water content was calculated based on the following equation:

$$\text{Relative water content [\%]} = \left(\frac{\text{FW-DW}}{\text{SW-DW}}\right) \times 100$$

where FW = Fresh weight (g), DW = Dry weight (g), SW = Saturated weight (g)

Electrolyte leakage (EL)

Electrolyte leakage percentage was measured following Jiang and Huang (2002). Fresh rice leaves were cut into 1 cm lengths (6 pieces) and transferred into a test tube containing 10 ml of deionised water, kept at room temperature for 24 h, and then measured for electrical conductivity as EC1. The solution containing the plant samples was then autoclaved for 15 min at 121°C followed by a second conductivity measurement as EC2 at room temperature. The following formula was used to determine the percentage of electrolyte leakage:

$$\text{Electrolyte leakage (\%)} = (EC1 / EC2) \times 100$$

Malonaldehyde

Malonaldehyde (MDA) was studied based on Sunohara and Matsumoto (2004). One gram of leaves (FW) was ground with 10 ml of 0.1% (w/v) trichloroacetic acid (TCA) and then centrifuged at 4°C for 5 min (14,000 rpm). Two milliliters of supernatant were transferred to another test tube and heated for 25 min at 95°C with 9 ml of 0.5% (w/v) thiobarbituric acid (TBA). All reactions were stopped by chilling on ice for 10 min. The absorbance was determined at two wavelengths: 532 nm (A532) and 600 nm (A600) using 20% TCA as the blank. MDA content was calculated using the following equation:

$$\text{MDA (µmol/gFW)} = \frac{(A_{532} - A_{600}) \times Vf \times Ve}{155 \times Va \times FW}$$

where Vf = Final volume

Ve = Trichloroacetic acid (TCA) volume

Va = Solvent volume

FW = Plant sample fresh weight

Anatomical study

The middle part of mature rice leaves was transverse-sectioned by the free-hand section technique, while an abaxial epidermal inspection was conducted by the leaf peeling technique. The middle portion of the leaves was cut into small pieces. These pieces were then soaked in 15% (v/v) Clorox (sodium hypochlorite) for 24 h before peeling to remove the adaxial mesophyll. The transverse-sectioned and peeled samples were stained with 1% (w/v) safranin O in ethyl alcohol for 1-2 min, then dehydrated with serial ethyl alcohol and xylene and mounted by DePeX. All anatomical characteristics were observed and measured based on Nawazish *et al*. (2006), Zhang *et al*. (2015), and Taratima *et al*. (2019).

Statistical analysis

All data obtained from the experiments were averaged and analyzed for variance using one-way analysis of variance (ANOVA) and the Independent Samples t-Test. Differences in the mean values of each experimental group were compared using Scheffe's Test by the IBM SPSS Statistics version 28 program at a confidence level of 0.05. Pearson's correlation coefficient was performed among the growth, physiological, and anatomical parameters to reveal the collected parameter relationships. The data were also scaled and submitted to hierarchical cluster analysis (HCA) to cluster the treated rice, with results expressed as heatmaps based on growth, physiological, and anatomical parameters using Euclidean distance and average linkage. Pearson's correlation coefficient and HCA were carried out using R version 4.4.1 for Windows (Posit Software, USA).

Physiological and anatomical parameters from all treatments were scaled (Autoscaling) before submitting to PLS-DA analysis to cluster 'Tubtim Chumphae' rice treated with drought and rewatering conditions. OPLS-DA analysis was performed with the control treatment at 45 days and the drought treatment of 'Tubtim Chumphae' rice to cluster and identify the key parameters. Both PLS-DA and OPLS-DA analyses were conducted using MetaboAnalyst version 6.0.

Results

Soil moisture content and growth performance

The soil moisture analysis revealed substantial differences between the drought stress and control group treatments. The drought stress treatment had significantly lower soil moisture content than the control group but moisture contents were similar after rewatering (Table 1). Drought stress severely impacted the shoot and root growth of 'Tubtim Chumphae' rice (Figure 1), with shoot length significantly reduced under drought treatment, measuring 66.10 cm compared to 84.72 cm in the control group after 45 days ($p < 0.001$). By contrast, the effects of drought stress were not pronounced in root length and root dry weight ($p > 0.05$). Both fresh and dry weights of shoots and roots under drought condition were lower compared to the control group over the same period ($p < 0.001$) but exhibited a substantial increase after rewatering for 10 days ($p < 0.001$; Table 1).

Drought stress also hindered leaf growth, as evidenced by a significant reduction in leaf number per plant to 15.00, compared to 22.60 in the control treatment at 45 days ($p < 0.001$). Leaf width and length also decreased to 0.77 cm and 47.62 cm, respectively under drought stress compared to the control at 45 days ($p < 0.001$). The leaves of 'Tubtim Chumphae' rice displayed recovery effects after rewatering, especially in leaf length that extended to 59.60 cm and was not significantly different from the control treatment at 55 days ($p > 0.05$). Recovery effects due to rewatering were also observed in leaf number and leaf width, which significantly increased to 24.00 and 1.30 cm, respectively ($p < 0.001$; Table 1).

Growth during the tillering stage, as indicated by tiller number per plant and circumference, was also influenced by drought stress. The number of tillers per plant and circumference significantly reduced from 7.00 and 5.08 cm in the control treatment at 45 days to 5.00 and 3.58 cm after exposure to drought stress, respectively ($p < 0.001$). Tiller number and circumference increased to 6.80 and 6.40 cm after the rewatering

treatment (Table 1). The drought stress treatment group gave significantly greater values of leaf rolling and drought score than the control group. Leaf rolling score dramatically increased to 5.0 under drought stress compared to the control at 45 days and decreased to 2.2 after the rewatering treatment. Drought recovery score (DRS) measurements after rewatering for 10 days increased compared to the control (Table 1) with new leaves and clumps (Figure 1).

Table 1. Soil moisture content, growth characteristics of 'Tubtim Chumphae' rice under the control treatment at 45 and 55 days, drought treatment for 21 days and drought treatment with rewatering for 10 days

Characteristic	Control at 45 days	Drought stress treatment	Control at 55 days	Rewatering
Soil moisture content (%)	85.26 ± 5.35a	9.59 ± 0.16c	87.98 ± 4.86a	84.94 ± 3.12a
Shoot length (cm)***	84.72 ± 2.17b	66.10 ± 4.77c	101.36 ± 2.14a	88.52 ± 2.87b
Shoot fresh weight (g)***	14.27 ± 1.75c	6.36 ± 0.83d	34.15 ± 0.65a	21.17 ± 1.81b
Shoot dry weight (g)***	3.6 ± 0.42c	1.94 ± 0.27d	10.19 ± 0.50a	5.53 ± 0.52b
Root length (cm)*	39.08 ± 2.81b	41.64 ± 1.99ab	42.92 ± 1.54a	43.04 ± 2.47a
Root fresh weight (g)***	4.70 ± 0.65c	1.52 ± 0.12d	36.00 ± 1.27a	18.13 ± 0.91b
Root dry weight (g)***	0.67 ± 0.10c	0.40 ± 0.03c	6.59 ± 0.39a	3.46 ± 0.37b
Leaf number/plant***	22.60 ± 2.70b	15.00 ± 1.58c	28.20 ± 2.68a	24.00 ± 2.35b
Leaf width (cm)***	1.41 ± 0.07b	0.77 ± 0.08d	1.69 ± 0.07a	1.30 ± 0.07c
Leaf length (cm)***	62.52 ± 3.21a	47.62 ± 3.68b	63.24 ± 1.78a	59.60 ± 1.58a
Tiller number/plant***	7.00 ± 1.00b	5.00 ± 0.71c	9.00 ± 0.71a	6.80 ± 0.84b
Circumference (cm)***	5.08 ± 0.59c	3.58 ± 0.33d	7.78 ± 0.43a	6.40 ± 0.54b
Leaf rolling score	2.2 ± 0.83b	5.0 ± 0.00a	1.2 ± 0.44b	2.2 ± 0.44b
Drought score	1.2 ± 0.44bc	3.0 ± 0.70a	0.6 ± 0.54c	2.2 ± 0.44ab
DRS	-	-	1.2 ± 0.44b	3.0 ± 1.41a

Lowercase letters represent significant differences among the four treatments for the same parameter (row) with values exhibited as mean ± standard deviation (SD); * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, DRS = Drought recovery score

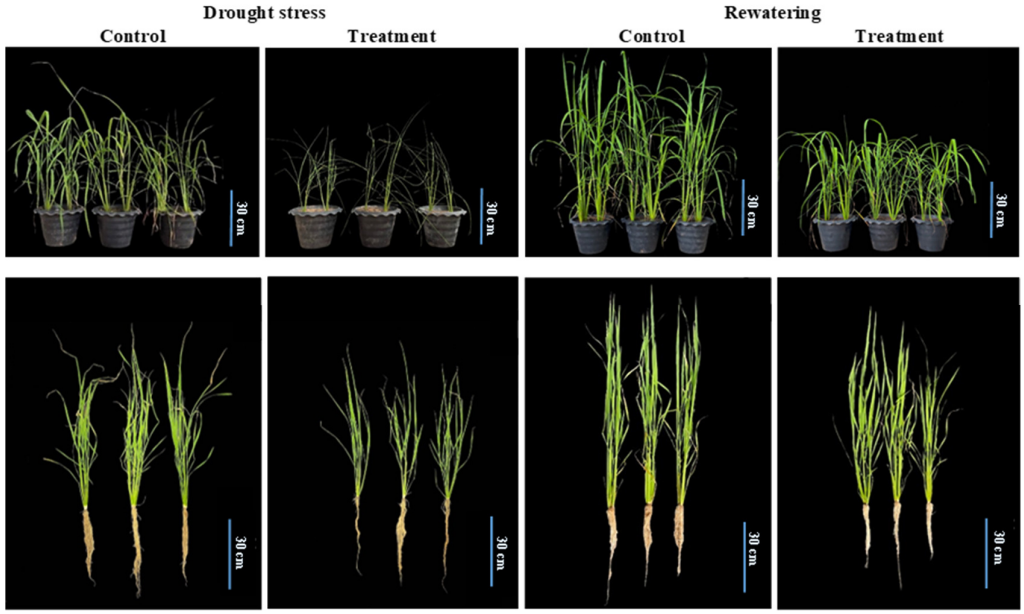

Figure 1. 'Tubtim Chumphae' rice plants under the control and drought stress treatments at 45 days (drought stress period) and 55 days (rewatering period)

Physiological responses

Dark-adapted chlorophyll fluorescence (Fv/Fm) was not significantly different across all treatments. However, light-adapted chlorophyll fluorescence (Fv'/Fm') of 'Tubtim Chumphae' rice after drought treatment exhibited a decrease compared to the control at 45 days. Fv'/Fm' was not influenced by rewatering and did not differ from the value (0.7) measured after drought treatment ($p > 0.05$). Drought treatment did not influence chlorophyll fluorescence but the SPAD value increased to 42.04 compared to 33.62 for the 45-day control treatment ($p < 0.001$). Interestingly, the SPAD value decreased after rewatering with an insignificant difference from the control at 45 and 55 days ($p > 0.05$). The similar response pattern was observed in the content of chlorophyll a, b, and total chlorophyll (Table 2).

The relative water content (RWC) was calculated to determine the water status in the leaves of 'Tubtim Chumphae' rice. Leaves exposed to drought stress exhibited a significant decrease in RWC to 76.44% ($p < 0.01$), while RWC values of the control at 45 and 55 days and the rewatering treatment varied between 83.11 and 86.52% (Table 2). The results indicated the recovery of leaf water status after rewatering. By contrast, malondialdehyde (MDA) content and electrolyte leakage, indicators for lipid peroxidation and membrane integrity, sharply increased under drought stress to 0.09 μmole/g FW and 31.16%, respectively compared to the control at 45 days ($p < 0.001$). After rewatering, these parameters decreased to 0.04 μmole/g FW and 26.73% with insignificant differences from the control at the same age ($p > 0.05$; Table 2).

Table 2. Physiological response of 'Tubtim Chumphae' rice under the control treatment at 45 and 55 days, drought treatment for 21 days and drought treatment with rewatering for 10 days

Characteristic	Control at 45 days	Drought stress treatment	Control at 55 days	Rewatering
Fv/Fm	0.77 ± 0.04a	0.76 ± 0.05a	0.79 ± 0.02a	0.76 ± 0.03a
Fv'/Fm'*	0.73 ± 0.03ab	0.70 ± 0.05b	0.76 ± 0.02a	0.70 ± 0.02b
SPAD value***	33.62 ± 2.10b	42.04 ± 1.54a	34.58 ± 1.33b	33.14 ± 1.73b
Chlorophyll a content (mg/g tissue)***	0.27 ± 0.05c	0.68 ± 0.06a	0.26 ± 0.08c	0.45 ± 0.07b
Chlorophyll b content (mg/g tissue)***	0.10 ± 0.02b	0.19 ± 0.06a	0.08 ± 0.02b	0.18 ± 0.04a
Total chlorophyll content (mg/g tissue)***	0.37 ± 0.07c	0.87 ± 0.09a	0.35 ± 0.01c	0.63 ± 0.08b
Relative water content (%)**	83.11 ± 1.86a	76.44 ± 4.17b	86.52 ± 4.55a	83.59 ± 3.20a
MDA content (μmole/g FW)***	0.04 ± 0.00b	0.09 ± 0.01a	0.04 ± 0.00b	0.04 ± 0.01b
Electrolyte leakage (%)**	24.60 ± 1.36b	31.16 ± 1.95a	26.78 ± 3.26b	26.73 ± 1.28b

Lowercase letters represent significant differences among the four treatments for the same parameter (row) with values exhibited as mean ± standard deviation (SD); * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

Anatomical changes

Leaf thickness varied between 16.93 and 18.76 μm, with no significant differences across all treatments ($p > 0.05$). However, under the microscope, the thickness of the cuticle and cell wall on the upper and lower epidermal side of the midrib (CCW-MR-U and -L) and lamina blade (CCW-MR-U and -L) increased in leaves under drought stress. Interestingly, the leaves anatomically responded to rewatering by a decrease in thickness with no significant difference compared to the control treatment at 55 days ($p > 0.05$; Table 3). By contrast, the size of the vascular bundles at the midrib (VB-MR), lamina blade (VB-LA), and small vascular bundles (VB-SM), indicated by vertical (V) and horizontal (H) lengths, significantly decreased under drought stress ($p < 0.01$). Rewatering treatment exhibited partial recovery of these vascular bundle size parameters, resulting in an increase compared to the drought treatment ($p < 0.01$; Table 3).

The number of bulliform cells markedly increased to 8.00 due to drought stress, while the control treatment at 45 and 55 days ranged between 5.00 and 5.20 cells ($p < 0.01$). Rewatering reduced the number of

bulliform cells to 5.60, which was not significantly different from the control treatment ($p > 0.05$). Despite the increase in the number of bulliform cells, drought stress caused a reduction in their size. The vertical length of the bulliform cells significantly reduced from 23.41 μm in the control treatment at 45 days to 16.21 μm under drought treatment ($p < 0.001$). Reduction in horizontal length was also observed under drought treatment. However, the size of bulliform cells significantly increased when the drought-treated rice was rewatered (Table 3). Stomata and epidermal cells responded to drought stress and rewatering in a manner similar to bulliform cells. These cells reduced in size under drought condition and then partially recovered after rewatering. The xylem area at the midrib and lamina blade showed similar patterns in response to drought, decreasing to 124.89 and 80.29 μm^2, respectively compared to the control at 45 days ($p < 0.001$). After rewatering, the areas increased to 373.90 and 227.30 μm^2 (Figure 2 and Table 3).

Table 3. Anatomical changes of 'Tubtim Chumphae' rice under the control treatment at 45 and 55 days, drought treatment for 21 days and drought treatment with rewatering for 10 days

Characteristic	Control at 45 days	Drought stress treatment	Control at 55 days	Rewatering
Leaf thickness (μm)	16.93 ± 1.33a	18.76 ± 1.94a	16.99 ± 0.54a	17.73 ± 0.92a
CCW-MR-U (μm)***	2.08 ± 0.25c	3.04 ± 0.29a	2.45 ± 0.23bc	2.80 ± 0.36ab
CCW-MR-L (μm)***	1.81 ± 0.12b	2.91 ± 0.24a	1.77 ± 0.50b	2.14 ± 0.34b
CCW-LA-U (μm)**	1.42 ± 0.28b	2.02 ± 0.19a	1.54 ± 0.12b	1.47 ± 0.23b
CCW-LA-L (μm)*	1.51 ± 0.26b	1.92 ± 0.21a	1.71 ± 0.11b	1.80 ± 0.20b
VB-MR-V (μm)***	60.04 ± 8.18b	42.91 ± 1.67c	72.68 ± 7.77a	61.77 ± 6.18b
VB-MR-H (μm)***	71.29 ± 10.18b	46.31 ± 0.76c	81.84 ± 6.13a	70.74 ± 5.00b
VB-LA-V (μm)**	43.06 ± 7.68a	32.59 ± 4.43b	52.63 ± 8.06a	46.73 ± 8.23a
VB-LA-H (μm)**	53.25 ± 6.65a	40.64 ± 6.95b	64.79 ± 12.33a	57.02 ± 5.64a
VB-SM-V (μm)	14.94 ± 0.99a	14.69 ± 1.75a	16.17 ± 0.14a	14.46 ± 1.56a
VB-SM-H (μm)***	13.20 ± 1.15bc	11.88 ± 2.31c	16.94 ± 1.34a	14.23 ± 0.45b
BCN (number)**	5.00 ± 1.00b	8.00 ± 0.71a	5.20 ± 0.84b	5.60 ± 1.52b
BC-L-V (μm)***	23.41 ± 1.10b	16.21 ± 0.95d	25.18 ± 1.59a	21.79 ± 0.70c
BC-L-H (μm)**	39.54 ± 2.89ab	33.20 ± 2.30c	41.51 ± 5.08a	35.33 ± 2.13bc
STW (μm)**	10.35 ± 1.66ab	9.20 ± 1.32c	12.28 ± 0.88a	11.81 ± 1.31bc
STL (μm)**	13.33 ± 1.60b	12.81 ± 1.47b	18.29 ± 1.33a	16.75 ± 0.79a
ECW (μm)*	10.59 ± 1.33ab	9.01 ± 1.77b	12.46 ± 1.09a	9.77 ± 1.52b
ECL (μm)	47.60 ± 6.40a	39.90 ± 6.18a	48.65 ± 5.76a	47.74 ± 3.07a
X-AREA-MR (μm^2)***	374.09 ± 16.52b	124.89 ± 3.09c	437.37 ± 49.19a	373.90 ± 6.41b
X-AREA-LA (μm^2)***	213.17 ± 6.66b	80.29 ± 13.27c	325.65 ± 43.25a	227.30 ± 38.50b

Lowercase letters represent significant differences among the four treatments for the same parameter (row) with values exhibited as mean ± standard deviation (SD); * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

CCW-MR = cuticle and cell wall thickness of midrib; CCW-LA = cuticle and cell wall thickness of lamina blade; VB-MR = vascular bundle length at midrib; VB-LA = vascular bundle length at lamina blade; VB-SM = length of small vascular bundle; BCN = bulliform cell number; BC-L; length of bulliform cells; STW = stomatal width; STL = stomatal length; ECW = epidermal cell width; ECL = epidermal cell length; X-AREA-MR = xylem area at midrib; X-AREA-LA = xylem area at lamina blade; U = upper epidermal side, L = lower epidermal side; V = vertical; H = horizontal

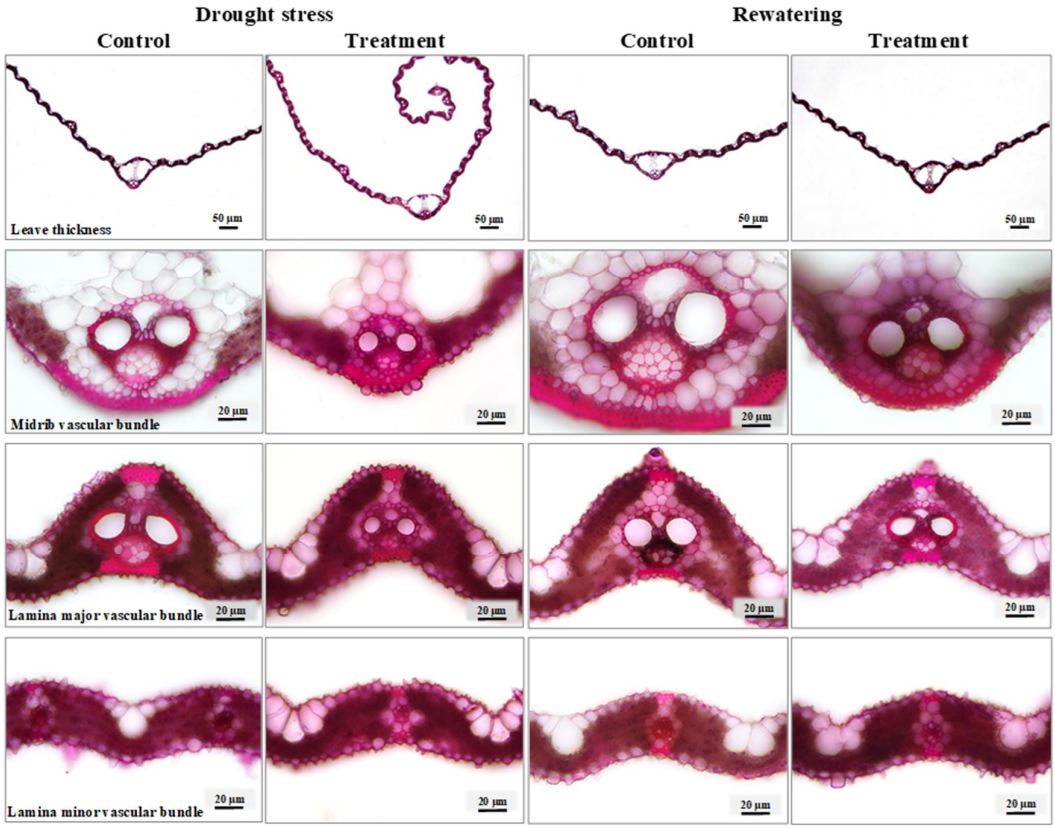

Figure 2. Microscopic analysis of 'Tubtim Chumphae' rice leaves under the control (45 and 55 days), drought and rewatering treatments

Pearson's correlation coefficient and hierarchical cluster analysis (HCA)

The relationships between the measured parameters were analyzed using Pearson's correlation coefficient. The results revealed a significantly positive correlation among growth parameters ($p < 0.001$) except for root length (RL) which did not exhibit significant correlation with other growth parameters ($p > 0.05$). The growth parameters also positively correlated with the anatomical parameters except for cuticle and cell wall thickness and number of bulliform cells that displayed a negative correlation ($p < 0.001$). By contrast, physiological parameters, excluding chlorophyll fluorescence values which positively correlated with each other within their group, exhibited a significantly negative correlation with all growth parameters and most anatomical parameters, except for cuticle and cell wall thickness, and the number of bulliform cells ($p < 0.001$). Considering the anatomical parameters, size of vascular bundle, bulliform cells, stomata, epidermal cells, and xylem area negatively correlated with the number of bulliform cells, cuticle and cell wall thickness, and most physiological parameters, while strongly and positively correlating with growth parameters ($p < 0.001$; Figure 3).

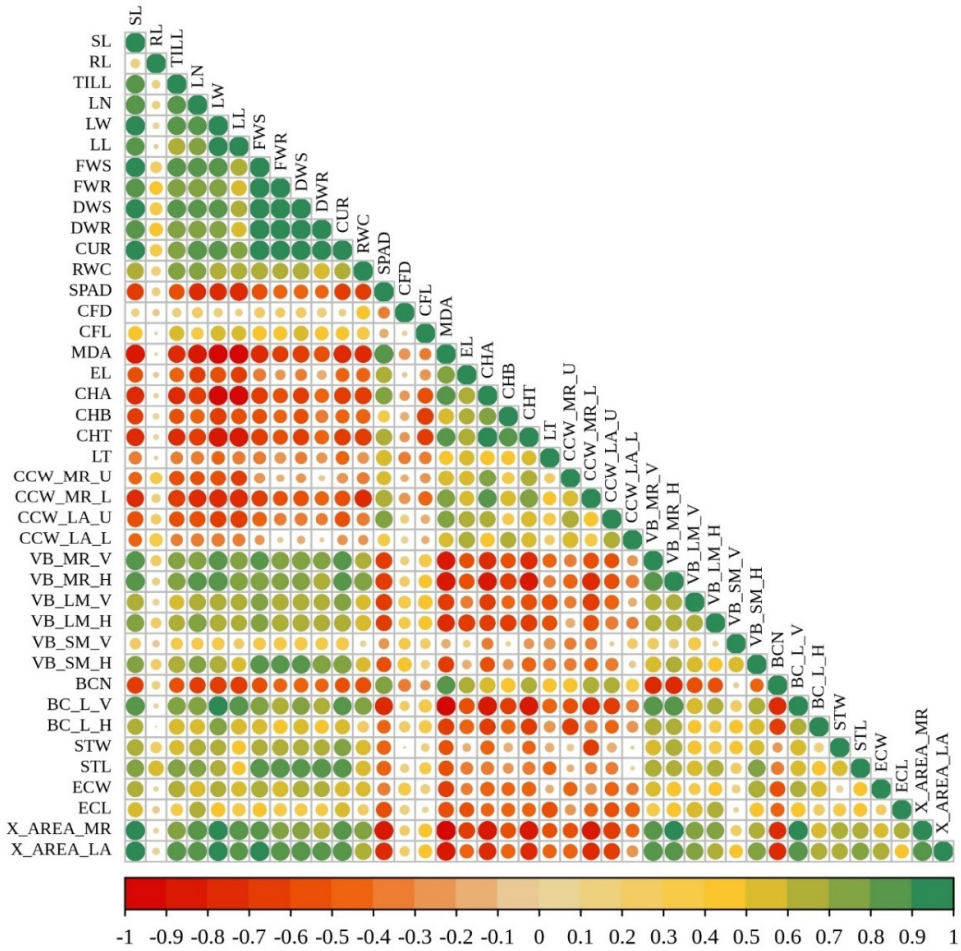

Figure 3. Pearson's correlation coefficient results among growth, physiological, and anatomical parameters of 'Tubtim Chumphae' rice in the control, drought, and rewatering treatments
CCW-MR = cuticle and cell wall thickness of midrib; CCW-LA = cuticle and cell wall thickness of lamina blade; VB-MR = vascular bundle length at midrib; VB-LA = vascular bundle length at lamina blade; VB-SM = length of small vascular bundle; BCN = bulliform cell number; BC-L; length of bulliform cells; STW = stomatal width; STL = stomatal length; ECW = epidermal cell width; ECL = epidermal cell length; X-AREA-MR = xylem area at midrib; X-AREA-LA = xylem area at lamina blade; U = upper epidermal side, L = lower epidermal side; V = vertical; H = horizontal.

Hierarchical cluster analysis (HCA) accompanied by a heatmap exhibited the response patterns of the four experimental treatments in all groups of measured parameters. 'Tubtim Chumphae' rice in the control group and treatments of drought and rewatering were separated into three major clusters. Cluster I contained rice treated under drought stress. This cluster displayed high expression levels in cuticle and cell wall thickness (CCW), chlorophyll content (CHA, CHB, and CHT), electrolyte leakage (EL), number of bulliform cells (BCN), MDA content, and SPAD value while the other parameters were expressed at lower levels. The control treatment at 45 days and the rewatering treatment were grouped in cluster II, where all parameters were expressed at moderate levels. Parameters that were highly expressed in cluster I declined in cluster III and included the control treatment at 55 days. However, the other treatments, which were all growth parameters, relative water content (RWC), vascular bundle size (VB), stomatal size (STL and STW), leaf thickness (LT), bulliform cell size (BCN), and epidermal cell size (ECL and ECW) increased in expression levels compared to clusters I and II (Figure 4).

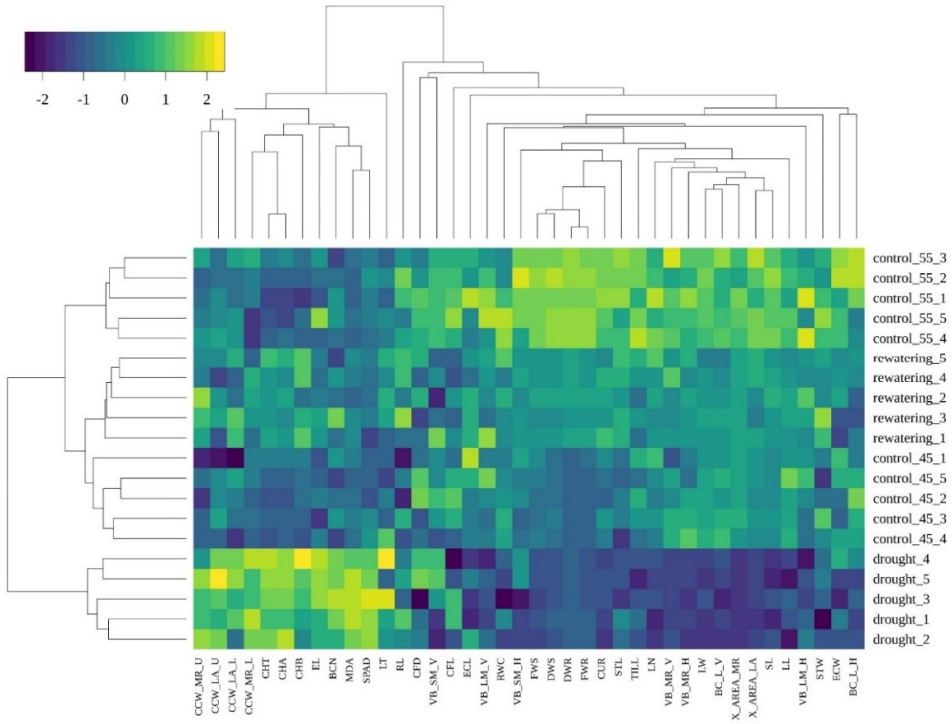

Figure 4. Hierarchical cluster analysis (HCA) representing clusters of 'Tubtim Chumphae' rice based on growth, physiological, and anatomical parameters that responded to drought and rewatering treatments CCW-MR = cuticle and cell wall thickness of midrib; CCW-LA = cuticle and cell wall thickness of lamina blade; VB-MR = vascular bundle length at midrib; VB-LA = vascular bundle length at lamina blade; VB-SM = length of small vascular bundle; BCN = bulliform cell number; BC-L; length of bulliform cells; STW = stomatal width; STL = stomatal length; ECW = epidermal cell width; ECL = epidermal cell length; X-AREA-MR = xylem area at midrib; X-AREA-LA = xylem area at lamina blade; U = upper epidermal side, L = lower epidermal side; V = vertical; H = horizontal.

PLS-DA analysis of physiological and anatomical parameters

A partial least squares-discriminant analysis (PLS-DA) was performed to classify 'Tubtim Chumphae' rice based on the physiological and anatomical parameters, with predefined groups as the control treatment at 45 and 55 days, drought, and the rewatering treatment. The results of PLS-DA were exhibited as a score plot generated from the first two components responsible for 51.1% and 9.7% of the explained variance, respectively (Figure 5A), displayed as four distinct groups. The treatments of rewatering and the control at 45 and 55 days were grouped together while 'Tubtim Chumphae' rice under drought treatment was separate. A loading plot indicated that 12 parameters were influenced by drought treatment as the cuticle and cell wall thickness at the lamina blade and midrib (CCW-LA-L, CCW-LA-U, CCW-MR-U, and CCW-MR-L), chlorophyll content (CHA, CHB, and CHT), electrolyte leakage (EL), leaf thickness (LT), number of bulliform cells (BCN), and MDA content. The other treatments were grouped based on 17 other parameters (Figure 5B).

The variable importance in projection (VIP) plot highlighted the 15 most crucial parameters for differentiating 'Tubtim Chumphae' rice across all treatments, based on their expression levels (Figure 5C). The plot showed that 6 parameters provided more than 1.2 VIP score. These were cuticle and cell wall thickness on the upper side of the midrib (CCW-MR-U), chlorophyll b (CHB), total chlorophyll content (CHT), chlorophyll a (CHA), horizontal length of bulliform cells (BC-L-H), and cuticle and cell wall thickness on the lower side of the lamina (CCW-LA-L). Considering CCW-MR-U, CHT, CHA, and CCW-LA-L, 'Tubtim Chumphae' rice treated under drought stress exhibited the highest expression levels compared to the other treatments (Figure 5D, 5F-G, 5I). The chlorophyll b content in the drought treatment was higher than in the

control treatment but was the same as in the rewatering treatment (Figure 5E). Bulliform cells in the control treatment and rewatering treatment were horizontally longer than bulliform cells in the drought treatment (Figure 5H), indicating that drought stress strongly influenced bulliform cell size.

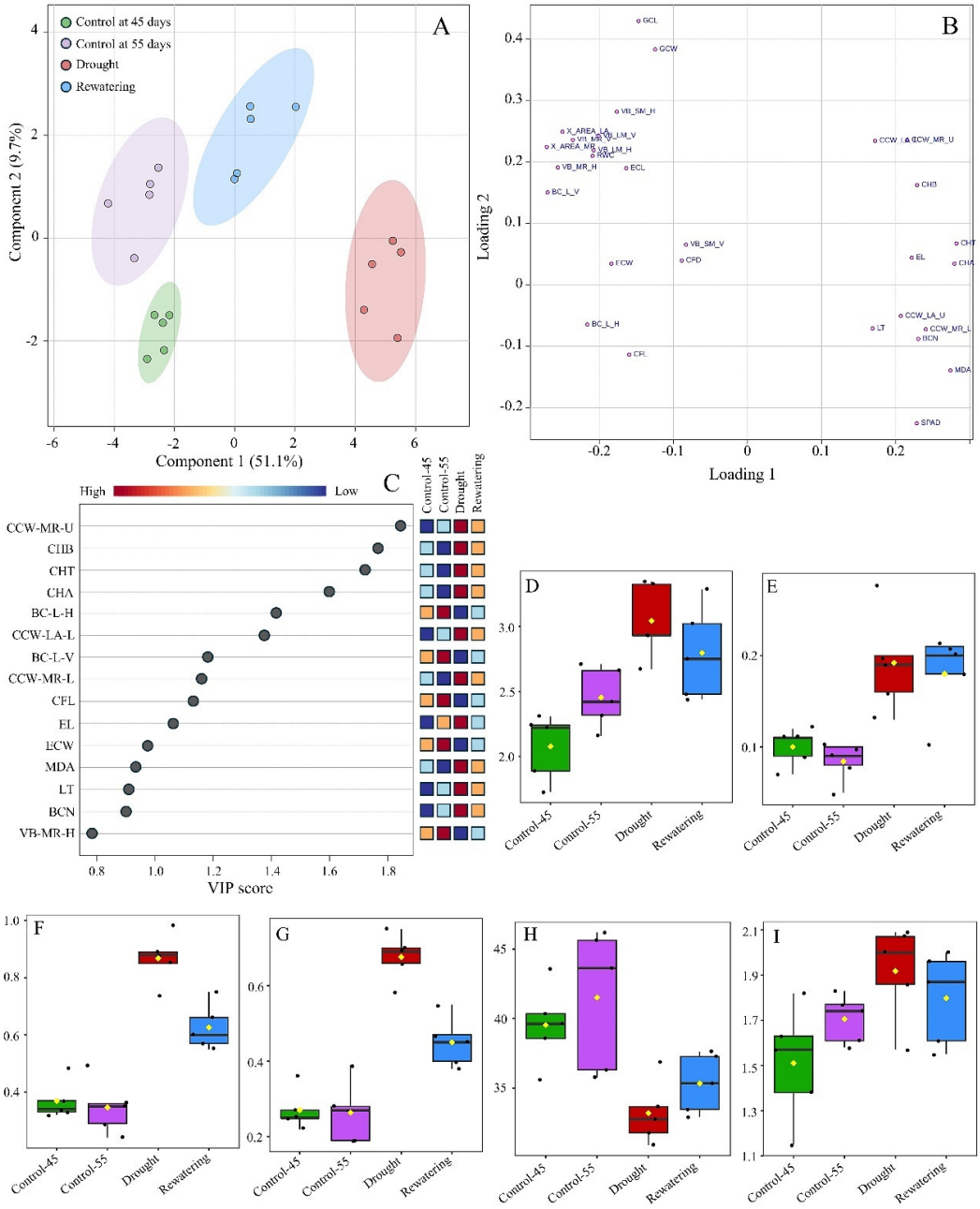

Figure 5. PLS-DA results of 'Tubtim Chumphae' rice in the treatments of control (45 and 55 days), drought, and rewatering

(A) A score plot exhibiting rice clusters based on the recorded parameters. (B) A loading plot of physiological and anatomical parameters. (C) A VIP plot showing 15 crucial parameters. (D) A boxplot of cuticle and cell wall thickness on the upper side of the midrib. (E) A boxplot of chlorophyll b content. (F) A boxplot of chlorophyll a content. (G) A boxplot of total chlorophyll content. (H) A boxplot of the horizontal length of bulliform cells. (I) A boxplot of cuticle and cell wall thickness on the lower side of the lamina.

OPLS-DA analysis between the control and drought treatments

The physiological and anatomical parameters were analyzed using orthogonal partial least squares-discriminant analysis (OPLS-DA) to cluster 'Tubtim Chumphae' rice predefined as normal (control at 45 days) and drought treated rice to identify the potential physiological and anatomical markers for drought stress. The score plot of OPLS-DA (Figure 6A) exhibited two distinct groups as the control group at 45 days (green) and the drought group (red). The model overview and permutation test (n = 2000) results showed a significant number of clusters ($p < 0.01$) with R2Y = 0.99, and the reliability of the model was indicated by more than 0.95 Q2. The VIP score plot displayed 15 crucial parameters that influenced rice clustering. The parameters in descending order were xylem area in the midrib and lamina (X-AREA-MR and X-AREA-LA), MDA content, vertical length of bulliform cells (BC-L-V), chlorophyll a (CHA), total chlorophyll content (CHT), cell wall thickness on the lower side of the midrib (CCW-MR-L), and SPAD value (Figure 6B). Eight parameters gave a VIP score higher than 1.2. The expression levels of X-AREA-MR, X-AREA-LA, and BC-L-V were distinctly elevated in the control treatment at 45 days (Figure 6C-7D, 7F), while MDA content, CHA, CHT, CCW-MR-L, and SPAD value were highly expressed in the treatment of drought stress compared to the control (Figure 6E, 6G-6J). The OPLS-DA clustered the rice from the control and drought treatments and identified the potential markers for drought stress in 'Tubtim Chumphae' rice.

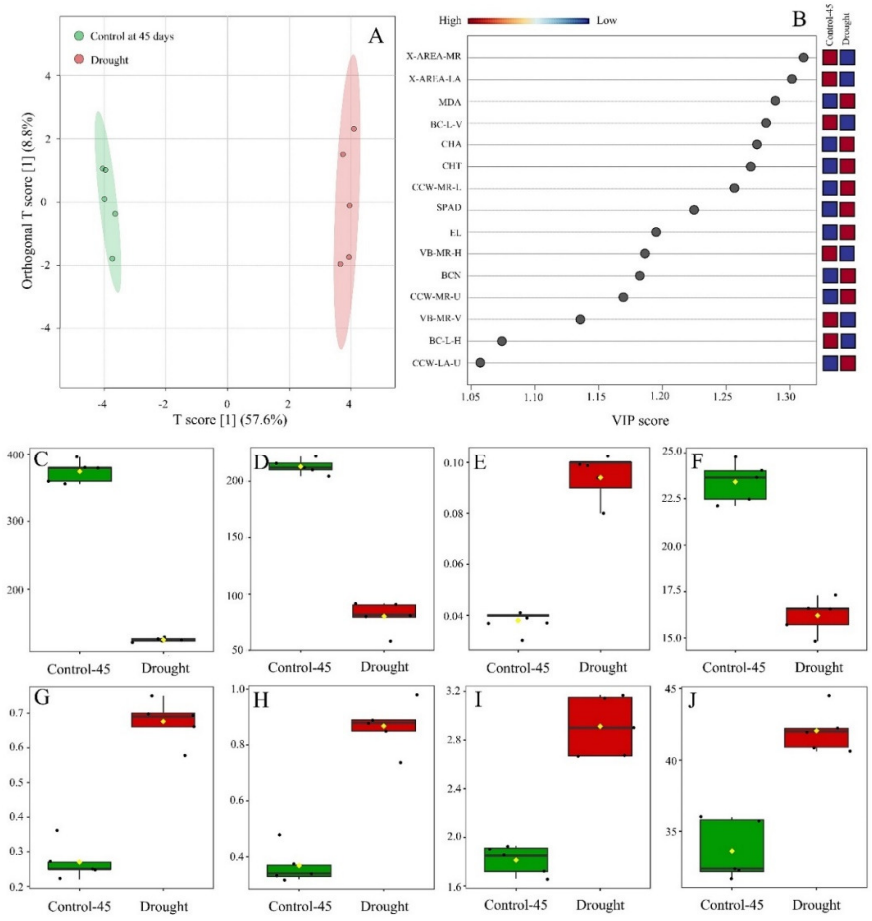

Figure 6. OPLS-DA results of 'Tubtim Chumphae' rice in the treatments of control at 45 days, and drought (A) A score plot exhibiting the clusters of control and drought treatments (B) A VIP plot showing 15 crucial parameters. (C-D) Boxplots of the xylem area in the midrib and lamina. (E) MDA content. (F) A boxplot of the vertical length of bulliform cells. (G) A boxplot of chlorophyll a content. (H) A boxplot of total chlorophyll a content. (I) A boxplot of cuticle and cell wall thickness on the lower side of the midrib. (J) A boxplot of the SPAD value.

Discussion

Our findings showed that 'Tubtim Chumphae' rice growth was significantly reduced by drought stress, concurring with previous reports (Moonmoon and Islam, 2017; Evamoni *et al*., 2023). The treatment group that lacked water had lower root fresh and dry weight than the control group but longer root length because plants induce root growth to discover deep groundwater and strengthen their resistance to drought (Zhou *et al*., 2020). Roots are the first organs to be impacted by changes in soil moisture under drought stress and they respond at morphological, anatomical, and molecular levels (Amtmann *et al*., 2022). Plants differ substantially in their root systems depending on the environment or their habitat (Schenk and Jackson, 2002). Drought-resistant species usually have deep roots, whereas drought-sensitive species tend to have shallow roots (Zhou *et al*., 2020). Roots react to drought stress differently depending on moisture source, culture condition, and plant species (Schenk and Jackson, 2002; Ogura *et al*., 2019; Kou *et al*., 2022).

Rice plants normally respond to primary drought stress by the wilting and curling of leaf margins. This is a mechanism for reducing leaf area to prevent water loss (Yavas *et al*., 2024). The treatment group had significantly higher values of leaf rolling than the control group, while leaf drought scores of the control group were higher than the treatment group. 'Tubtim Chumphae' rice has high susceptibility to leaf blight disease and brown planthopper attack (Changsri *et al*., 2016). Drought recovery score (DRS) measurements after rewatering for 10 days showed that the treatment group produced fewer new leaves and clumps than the control group because the treated rice was unable to replenish cells as usual during rewatering and the leaves that curled excessively recovered more slowly (Bunnag and Pongthai, 2013).

The relative water contents in the leaves of the treatment group were significantly lower than in the control group, indicating that drought stress affected the water status in plant cells (Moradi, 2016) with other physiological responses such as leaf curling to reduce transpiration and retain water. The relative water content value can be used as an important variable to determine drought stress tolerance (Bunnag and Pongthai, 2013), reflecting the balance between the amount of water transported to leaf tissue and the leaf transpiration rate, or the maximum amount of water that can be stored when fully developed (Lugojan and Ciulca, 2011).

Leaf green intensity as the SPAD value and chlorophyll content from this study significantly increased under drought treatment compared to the control, caused by loss of cell turgor. When plant leaves stop growing or leaf area decreases, chlorophyll density per leaf area increases (Taratima *et al*., 2021, Zhang *et al*., 2023). This can occur as a short-term effect, consistent with Rolando *et al*. (2015) who found that potato plants grown under limited water supply had a short-term increase in leaf greenness associated with cessation of leaf growth. In our study, chlorophyll fluorescence measurements of the treatment group decreased but were not statistically different from the control group under both light (Fv'/Fm') and dark condition (Fv/Fm). Drought stress reduced the electron transport efficiency of photosystem II (PSII), with less activity at the reaction center because the light energy conversion efficiency of PSII decreased (Wang *et al*., 2021) and caused damage to the photosynthetic pigments (Fu and Huang, 2001). High chlorophyll content in the leaves is not the only factor that contributes to high photosynthetic rate. Other factors that influence photosynthetic rate include light intensity, pH, carbon dioxide absorption and temperature (Dela Cruz, 2021). Drought stress also impacted electrolyte leakage (EL) and malondialdehyde (MDA), which were significantly higher than the control group. This indicated that lipid peroxidation caused cell membrane damage. Accumulation of MDA in cells limits the activity of antioxidant enzymes and accelerates cell death, resulting in electrolyte leakage, as reported in other rice genotypes (Asma *et al*., 2021) and wheat seedlings (Amoah *et al*. (2019).

The effect of drought stress on the anatomical changes in 'Tubtim Chumphae' rice in this study showed that cell turgor and expansion decreased, resulting in the reduction of xylem area and vascular bundle size both at the midrib and lamina blade which slowed down plant growth (Bunnag and Pongthai, 2013). However, leaf thickness and cuticle and cell wall thickness both in the midrib and lamina blades increased because the plants adapted to prevent evaporation and maintain water in their cells. These results concurred with Taratima *et al*.

(2021), who reported on the anatomical adaptations of sugarcane grown under drought stress by increasing the thickness of the leaf and cuticle layer while reducing vascular bundle size to limit the occurrence of air bubbles clogging the vascular bundle, thereby making water transport more efficient. Bulliform cell numbers increased under drought stress and during the rewatering period but their size was smaller than in the control group. Bulliform cells are large; they are filled with water but have thin walls. When plants are under drought stress, bulliform cells lose water, causing the cells to wither and become smaller. This correlated with the occurrence of leaf rolling due to the contraction of bulliform cells (Alvarez *et al*., 2008). The stomatal size of 'Tubtim Chumphae' rice decreased after drought stress treatment and rewatering, resulting in less absorption of CO_2 (Nawazish *et al*., 2006). The stomata play an important role in minimizing the amount of water loss, relating to the transpiration process that influences the flow of gases between the environment and the leaf, particularly CO_2 for photosynthesis (Lawson and Blatt, 2014).

Conclusions

Drought stress conditions inhibited the growth of 'Tubtim Chumphae' rice, causing changes in development and physiological characteristics. The relative water content decreased resulting in wilted, curled, and dry leaves while increases in electrolyte leakage percentage and malondialdehyde content indicated cellular damage. To preserve water in the cells and the rate of transpiration, rice must adapt to water deficit conditions by increasing the root length as well as reducing the size and number of leaves. 'Tubtim Chumphae' rice adapted to retain water in its cells by increasing leaf thickness, with a thicker cuticle layer and cell wall, increasing the number of bulliform cells, reducing vascular bundle size, and reducing the size of bulliform cells and stomata. These adaptations prevent water loss by plants. 'Tubtim Chumphae' rice seedlings adapted to withstand drought stress conditions. Our results can be applied for practical planning of 'Tubtim Chumphae' rice cultivation and other rice varieties to maximize yield in unpredictable climatic conditions.

Authors' Contributions

Conceptualization WT and NK; Data curation AT, CK, PM and PM; Formal analysis AT, CK, PM and PM; Funding acquisition WT; Investigation CK, PM and PM; Methodology WT and CK; Project administration WT; Resources CK, PM and PM; Software WT, AT and CK; Supervision WT and NK; Validation WT, AT and NK; Visualization WT and AT; Writing - original draft WT and AT; Writing - review and editing WT and AT. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

The authors thank the Department of Biology, Faculty of Science, Khon Kaen University for providing the laboratory facilities for this research. This study was funded by the KKU Research and Graduate Studies, Khon Kaen University (Research Program RP 67-10-001).

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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