



Evaluation of near-isogenic lines of rice introgressed with submergence tolerance and deep rooting at early growth stage

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Abstract Rice varieties that minimise shoot elongation under submergence and provide tolerance for up to two weeks carry *SUB1A*. Conversely, *DRO1* responsible for deep-rooting, helps in water and nutrient acquisition under drought. In this study, we compared the growth of NIL-SUB1, NIL-DRO1, and NIL-SUB1DRO1 with IR64 background for its dual tolerance to submergence, drought, and recovery. Submergence was on 14- and 16-days old seedling for 7 and 10 days and allowed to recover for 7

and 10 days respectively. Drought was also imposed on 14- and 13-days old seedlings for 18 and 29 days followed by 10 days recovery respectively. Submergence and drought adversely affected growth and performance of the genotypes. Compared to IR64, NIL-SUB1DRO1 suppressed shoot elongation during flooding, maintained the function of the leaf photosystem, and under drought conditions, maintained leaf water potential and contributed to increased dry matter weight of shoots and roots. In addition, NIL-SUB1DRO1 exhibited deep rooting, with roots extending into the lower subsurface layers under drought condition. It is suggested that NIL-SUB1 showed potential tolerance to submergence and NIL-DRO1 to drought. Furthermore, NIL-SUB1DRO1 was found to be tolerant to both drought and flooding.

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Introduction

Rice is a primary food source worldwide, providing food and livelihood security to half of the global human population (Samal et al. 2018). By 2035, rice production needs to increase by 26% to feed the growing population (Seck et al. 2012). As a semi-aquatic crop, rice faces various biotic and abiotic stresses due to different climatic, hydrologic, and

edaphic conditions. The increasing incidence extreme of abiotic stresses due to climate change is a significant constraint to meeting rising food demand and achieving global food security (Lesk et al. 2016). Approximately 30% of the world's rice (*Oryza sativa*) is grown at low elevations and depends on rainfall (Bailey-Serres et al. 2010). Rainfed farming reduces groundwater depletion, water pollution, and soil salinisation, which are often associated with controlled irrigation systems. However, rainfed fields are susceptible to flooding and drought due to inadequate water management. These two abiotic stresses are the most prevalent factors reducing rice yield in rainfed environments, affecting approximately 40 million hectares of rice at various crop stages globally and severely impacting plant growth, development, and yield (Barnabás et al. 2008). High rainfall over short periods can lead to flooding and low or no rainfall can lead to prolonged dry spells, significantly reducing crop yields (Lobell et al. 2011). In some cases, both floods and droughts may occur within the same season at different crop growth stages. In the coming years, rainfed shallow lowland areas will likely experience heavy precipitation during early crop growth stages, resulting in floods followed by dry spells causing drought. Variability in rainfall patterns, intensity and frequency due to climate change are major factors contributing to the unpredictable occurrence of drought and flood conditions.

Submergence leads to low oxygen availability in plants, obstructing aerobic respiration and hindering growth processes. In extreme cases, it can even result in plant death. Photosynthesis, crucial for supplying carbohydrates and O_2 for aerobic respiration, relies on CO_2 and irradiation (Setter et al. 1989). However, both CO_2 availability and irradiation are severely reduced by submergence. In water, CO_2 is limited due to its lower absolute concentration compared to O_2 . For instance, air-saturated water contains $0.268 \text{ mol m}^{-3} O_2$ but only $0.011 \text{ mol m}^{-3} CO_2$ at $25^\circ C$ and it diffuses slowly through the boundary layer (Jackson and Ram 2003). Additionally, reduced irradiance underwater, especially in turbid floodwater, further inhibits photosynthesis (Setter et al. 1987; Das et al. 2009) and significantly reduces chlorophyll content (SPAD value) and chlorophyll fluorescence (Fv/Fm) of susceptible genotypes (Nurrahma et al. 2021b). Certain rice cultivars exhibit distinct growth control strategies to survive submergence. One such

strategy is the quiescence syndrome (Colmer and Voesenek 2009), where shoot elongation is suppressed early in growth to conserve carbohydrates for an extended period (10–14 days) during flash floods. This response is regulated by the *SUB1A* gene, a major quantitative trait locus (QTL) responsible for submergence tolerance. FR13A, a highly tolerant rice landrace from Odisha, India, has been extensively studied for its *SUB1A*-mediated tolerance (Xu et al. 2006; Ismail et al. 2013; Panda and Sarkar 2012). Submergence tolerant cultivars can resume growth during desubmergence by utilising stored carbohydrates. Another survival strategy is the escape syndrome, observed in deepwater rice cultivars (Bailey-Serres and Voesenek 2008; Colmer and Voesenek 2009). These cultivars rapidly elongate leaf sheaths and internodes when faced with prolonged deep flooding. By doing so, they rise above the water surface, aided by the genes *SK1* and *SK2* (Hattori et al. 2011).

Drought stress induces a reduction in plant height, leaf area, and biomass (Mishra and Panda 2017). Leaf growth decreases due to limited water potential (Zhu et al. 2020), as disrupted water flow from the xylem to adjacent cells decreases turgor pressure, leading to impaired cell development and reduced leaf area in crops (Hussain et al. 2018). Characteristics such as leaf rolling and early senescence initiation are notable under drought stress (Anjum et al. 2011). Water scarcity adversely affects essential physiological traits of rice, including net photosynthetic rate, transpiration rate, stomatal conductance, water use efficiency, internal CO_2 concentration, photosystem II (PSII) activity, relative water content, and membrane stability index (Zhu et al. 2020; Melandri et al. 2021). Several factors contribute to the decline in photosynthesis, such as stomatal closure, reduced turgor pressure, decreased leaf gas exchange, and reduced CO_2 assimilation, ultimately damaging the photosynthetic apparatus (Gupta et al. 2020). Photosynthetic capacities of leaves and water availability in the root zones are pivotal in controlling growth and yield in susceptible rice genotypes under drought conditions (Zhu et al. 2020). Drought tolerance refers to the ability of plants to survive under low tissue water content (Kumar et al. 2017). Adaptive mechanisms like maintaining higher leaf water potential, better osmotic adjustment or protective actions such as

leaf rolling and stomatal closure are associated with plants' drought tolerance (Tuberosa 2012). Root characteristics play a crucial role in plant adaptation to drought stress. Crop performance under water stress depends on the root system characteristics of the variety used. Predicting rice production under water stress can be facilitated by considering root biomass (dry) and length (Comas et al. 2013). Root growth characteristics exhibit diverse responses under water stress, with rice varieties possessing deep and prolific root systems showing better adaptability, especially in deeper soils (Kim et al. 2020). Uga et al. (2011) found that a rice variety carrying the *DRO1* gene develops deeper and better root distribution under relatively dry upland conditions. They established a significant positive relationship between the *DRO1* gene and panicle weight, suggesting that *DRO1* enhances drought avoidance under natural field conditions with occasional water stress by promoting root elongation to access water and sustain essential physiological processes.

As sessile organisms, plants must cope with submergence and drought stress at some point in their life cycle. In this study, we evaluated the effectiveness of single genes and combined genes to enhance the tolerance to flooding and drought which are occurring singly, or both are expected to occur within a single growing season due to climate change, using new rice genotypes NIL-SUB1, NIL-DRO1, and NIL-SUB1DRO1. We assessed several morpho-physiological characteristics to understand and explain NIL-SUB1, NIL-DRO1, and NIL-SUB1DRO1 response to submergence and drought stress. IR64, FR13A, and Kinandang Patong were the parental varieties used to develop these genotypes. The availability of a rice genotype such as NIL-SUB1DRO1, which has the submergence and drought tolerance genes would be an advantage in the event that submergence and drought occurs within a single growing season. The results of this research would not only contribute significantly to breeding more resilient rice varieties but also expedite the deployment and dissemination of these genotypes to farmers in the future to sustain their productivity under the current worsening climate conditions.

Materials and methods

Plant materials

IR64-SUB1 (NIL-SUB1) resulted from a cross between IR64 (bred at IRRI, Philippines) and FR13A (Submergence tolerance rice) (Bailey-Serres et al. 2010), while IR64-DRO1 (NIL-DRO1) was developed at NARO in Japan through a cross between IR64 and Kinandang Patong (Drought tolerance rice variety). By crossing IR64-SUB1 and IR64-DRO1, we generated IR64-SUB1-DRO1 (NIL-SUB1DRO1) genotype through marker assisted molecular breeding with IR64 as their genetic background. NIL-SUB1, NIL-DRO1, NIL-SUB1DRO1, and IR64 are the genotypes used in this study.

Experiment 1-1, 1-2: submergence experiment

Cultivation conditions

As for the submersion experiment, the main experiment (1-2) was conducted following the preliminary experiment (1-1). First, in Experiment 1-1, we confirmed the growth of NIL-SUB1 and NIL-SUB1DRO1 relative to IR64 for the maximum quantum yield (Fv/Fm) and chlorophyll (SPAD value) in leaves, which is a typical indicator of flood tolerance under complete flood conditions. Next, in Experiments 1-2, in order to clarify the details of the anaerobic response of the NIL-SUB1DRO1 to IR64, the shoot elongation was also confirmed.

We studied two factors: environmental conditions, control and submergence treatments and varietal factors (NIL-SUB1DRO1, NIL-SUB1, and IR64 in Experiment 1-1, NIL-SUB1DRO1, and IR64 in Experiment 1-2). The experimental design followed a completely randomised approach replicated 5 times with 3 samples in each replication, with complete submergence for ten days followed 10 days of recovery in Experiment 1-1, while in Experiment 1-2, seven days of complete submergence followed by seven days of recovery,

In both submergence experiments, seeds from each genotype were placed in Petri dishes containing filter paper moistened with distilled water and left to germinate at 30 °C in an incubator under dark conditions for 24 h. The pre-germinated rice seeds were then sown in a commercial soil mix (N:P:K=0.9:2.3:1.1; pH

4.5–5.2) in the greenhouse. Sixteen-to fourteen-days-old seedlings for Experiment 1-1 and Experiment 1-2 were transplanted into hydroponic sponges (30 mm), which were inserted into seedling trays inside experimental glass containers (45 cm×45 cm×60 cm) in a controlled room of 12 h of light, with an intensity of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 12 h of dark, adjusted from light switch timer. The temperature in the room was set at 27 °C. A hydroponic solution (Hyponica culture solution, Kyowa Corporation, Japan) was maintained at 4.5 cm from the container base, the same height as the seedling tray and plant stem base, to acclimate the seedlings for four days in both experiments. The hydroponic solution was adjusted to pH 5.5. It contained 80 mg L⁻¹ N, 76 mg L⁻¹ P, 188 mg L⁻¹ K and other minor elements (Tada et al. 2014).

The complete submergence treatment was applied after the four days of acclimatisation by removing the hydroponic solution and supplying tap water to the transparent container box, reaching 45 cm above the plant shoot. For plants in the control treatment, tap water was maintained up to the stem base inside the transparent box. The submergence treatment lasted for ten days and 10 days of recovery in Experiment 1-1, while in Experiment 1-2, seven days followed by another seven days of recovery period during which the tap water was replaced with hydroponic solution up to the stem base for plants that received submergence and control treatments. Measurements of different variables were conducted before submergence, after submergence, after recovery periods in both experiments and after four days of complete submergence in Experiment 1-1.

Measurement of variables

Shoot length was measured from the base of the stem to the highest shoot tip using a ruler. The maximum quantum yield was measured after at least 2 h in the dark by clipping the chlorophyll fluorescence equipment (AquaPen-P AP-P 100, PSI, Czech Republic) onto a newly developed leaf of the sampled rice plant. A chlorophyll metre (SPAD-502, Konica Minolta Corporation, Japan) estimated the chlorophyll content (SPAD value) in leaves, with an average of three measurements taken on the upper part of the newly fully expanded leaf.

Experiment 2-1, 2-2: drought experiment

Cultivation conditions

Similarly, Experiment 2-2 aimed to evaluate useful traits associated with drought tolerance using the new rice genotype NIL-SUB1DRO1 compared to IR64 and assess their capacity to recover after stress release following preliminary Experiment 2-1. The Experiment 2-1 evaluated on tiller number and leaf area, the Experiment 2-2 evaluated photosynthetic traits and plant dry matter production.

The research was conducted in a greenhouse with mean temperature of 28 °C and a humidity of 55.2% in Experiment 2-1, while in Experiment 2-2 mean temperature was 25.4 °C and a humidity of 58.1%. Approximately 12 h of daylight was provided during the experimental periods.

The experiments involved two water status: drought treatment and a well-watered control. The experimental design followed a completely randomised approach replicated 5 times with 3 samples in each replication. Seeds from these genotypes were placed in petri dishes containing filter paper moistened with distilled water and left to germinate at 30 °C in an incubator under dark conditions for 48 h. The germinated seeds were carefully selected and directly sown into randomised PVC (polyvinyl chloride) pipes with an inner diameter of 8 cm and a height of 40 cm. The pipes were divided into two 20-cm layers: the A-layer (above 0–20 cm) and the B-layer (below 20–40 cm). Three seeds were initially sown per pipe and later thinned to one after five days. Each pipe was filled with a mixture of 2.6 kg of commercial soil (pH 4.5–5.2) and sandy soil in a 1:1 ratio for Experiment 2-1 and 1:4 ratio in Experiment 2-2. Additionally, 5 g of balanced compound fertiliser (N, P and K, 8-8-8) was added to each pipe.

The drought treatment began by stopping irrigation 13 days after transplanting in Experiment 2-1 (when seedlings were at 3.7–4.1 leaf age) and continued for 29 days. In contrast, daily irrigation was maintained for plants in the control treatment. In Experiment 2-2, drought treatment started 14 days after transplanting (when seedlings were at 3.7–4.1 leaf age) and lasted for 18 days. After the drought period in Experiment 2-2, daily irrigation resumed for both treatments, lasting for an additional 10 days as recovery period. Observations of variables were conducted before

drought treatment and after drought treatment in both experiments and after the recovery period in Experiment 2-2.

Measurement of variables

In the greenhouse, temperature and humidity were monitored using the long-range wireless connection logger telemoni TML2101-A (AS ONE Corporation, Japan). Soil moisture sensors placed at a depth of 10 cm provided continuous measurements of the soil moisture status in each pipe. Data were recorded using a ZL6 Basic Datalogger (Metre Group, Inc., Pullman, WA, USA) at 60-min intervals throughout the experiment. Stomatal conductance (gs) was measured daily on the newly developed leaf using a porometer (AP4, Delta-T Devices, Cambridge, UK) between 9:00 a.m. and 2:00 p.m. Shoot biomass and leaf area were determined by cutting the shoot and separating leaves and stems. Leaf area was quantified using digital image analysis software (LIA32, developed by Kazukiyo Yamamoto, Nagoya University). After measuring leaf area, leaves and stems were oven-dried at 80 °C for 48 h. to a constant weight before determining shoot dry weight. Roots from each layer were washed and stored in 70% ethanol at 4 °C for two weeks before root scanning. Root samples from each soil layer were scanned at 6400 dpi (EPSON XT-X830, Epson America Inc., Los Alamitos, CA, USA). The scanned images were analysed using an image analysis system (WinRHIZO, Regent Instruments Inc., QC, Canada) with a pixel threshold value ranging between 165 and 175 to assess root length, surface area and volume. After root analysis, root samples were oven-dried at 80 °C to a constant weight, following the same process as with the leaves and stems, to determine root dry weight. The number of tillers was obtained by physically counting the culms. Relative water content (RWC) was measured using fully expanded leaves between 9:00 a.m. and 2:00 p.m. Sampled leaves (5 cm length) were immediately weighed to determine leaf fresh weight (FW). They were then hydrated to full turgidity for four hours under normal room and light conditions. After hydration, the samples were blotted dry of surface water with tissue paper and weighed to obtain turgid weight (TW). Finally, the samples were oven-dried at 80 °C for 24 h and weighed to determine dry weight

(DW). RWC was calculated using the following formula (Barrs and Weatherley 1962).

$$\text{RWC \%} = \left[(\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \right] \times 100.$$

Leaf water potential was measured using a pressure chamber (WP4-T, METRE Group Inc., USA) between 9:00 a.m. and 2:00 p.m. Shoot length and SPAD values were assessed following the same procedure as in Experiment 1-1 and 1-2.

Data analysis

The data were analysed using a two-way analysis of variance. If significant differences were found, the least significant difference (LSD) test was performed at $p=0.05$ using IBM SPSS Statistics (Version 27.0.1.0).

Results

Effect of submergence on rice plant growth condition

Complete submergence for 10 and 7 days as well as drought conditions for 29 and 18 days negatively affected growth of the rice genotypes studied.

Experiment 1-1

Genotype and environment or submergence condition interaction was significant 10 days after complete submergence for SPAD value and Fv/Fm (Table 1). The interaction was also significant for SPAD value 4 days after complete submergence. The SPAD value and Fv/Fm of IR64 decreased significantly after 10 days of submergence compared to NIL-SUB1 and NIL-SUB1DRO1. The SPAD value of IR64 decreased by 47.3% compared to NIL-SUB1 and 45.3% for NIL-SUB1DRO1. There was no significant difference between these genotypes before submergence, 4 days after submergence and 10 days after desubmergence.

Experiment 1-2

The interaction of genotype and environment significantly influenced all parameters collected after submergence and recovery in Experiment 1-2

Table 1 Chlorophyll (SPAD value) and maximum quantum yield (Fv/Fm) of submergence Experiment 1-1

		0 DAS	4 DAS	10 DAS	10 DAR
<i>SPAD value</i>					
Control	IR64	32.5 ± 2.24 a	19.8 ± 4.03 a	11.7 ± 3.53 a	11.7 ± 4.14 a
	NIL-SUB1	30.4 ± 1.87 a	18.3 ± 1.48 a	15.2 ± 4.48 a	12.4 ± 3.40 a
	NIL-SUB1DRO1	32.1 ± 1.35 a	20.3 ± 2.52 a	11.4 ± 2.89 a	10.2 ± 3.89 a
Submergence	IR64		19.9 ± 4.61 A	9.8 ± 3.29 B	12.2 ± 5.87 A
	NIL-SUB1		20.6 ± 3.56 A	18.6 ± 2.88 A	14.1 ± 5.02 A
	NIL-SUB1DRO1		20.6 ± 1.80 A	17.9 ± 3.79 A	10.7 ± 3.92 A
ANOVA (<i>p</i> -value)	T		0.517 ns	0.019*	0.351 ns
	V		0.902 ns	< 0.001***	0.035*
	T*V		0.034*	0.005**	0.974 ns
<i>Maximum quantum yield (FvFm⁻¹)</i>					
Control	IR64	0.772 ± 0.018 a	0.811 ± 0.023 a	0.778 ± 0.019 a	0.796 ± 0.016 a
	NIL-SUB1	0.767 ± 0.015 a	0.810 ± 0.009 a	0.789 ± 0.003 a	0.787 ± 0.008 a
	NIL-SUB1DRO1	0.768 ± 0.018 a	0.807 ± 0.008 a	0.792 ± 0.017 a	0.788 ± 0.008 a
Submergence	IR64		0.802 ± 0.012 A	0.711 ± 0.041 B	0.791 ± 0.013 A
	NIL-SUB1		0.788 ± 0.011 A	0.766 ± 0.014 A	0.788 ± 0.006 A
	NIL-SUB1DRO1		0.783 ± 0.044 A	0.767 ± 0.016 A	0.784 ± 0.007 A
ANOVA (<i>p</i> -value)	T		0.003***	< 0.001***	0.087 ns
	V		0.14 ns	< 0.001***	0.033*
	T*V		0.523 ns	0.015*	0.186 ns

*Significant at $p < 0.05$, **significant at $p < 0.01$, ***significant at $p < 0.001$, and ns = not significant. Different letters in the same column indicate a significant difference at $p < 0.05$ according to the least significant difference (LSD) test. 0 DAS = 0 days after submergence, 4 DAS = 4 days after submergence, 10 DAS = 10 days after submergence, and 10 DAR = 10 days after recovery

(Table 2). There was no significant difference in shoot length between submerged IR64, control IR64 and NIL-SUB1DRO1 after submergence during the experiment (Fig. 1). While the shoot length of IR64 increased significantly (29.2%) when submerged, that of NIL-SUB1DRO1 did not change significantly (8.7%, ns) compared to the start of the submergence treatment. The slow growth characteristic conferred

by *SUB1A* under submergence was evident in the limited change in shoot length of NIL-SUB1DRO1, seemingly independent of the presence of DRO1. After 7 days of complete submergence, chlorophyll content (SPAD value) for IR64 decreased significantly (61.5%), whereas that for NIL-SUB1DRO1 decreased by only 12.7% (not significant) compared to the start of the submergence treatment (Fig. 2).

Table 2 Analysis of variance (ANOVA) of submergence Experiment 1-2

	Parameter	Collection date	Variety	Environment	Interaction (G X E)
*Significant at $p < 0.05$, **significant at $p < 0.01$, ***significant at $p < 0.001$, and ns = not significant. O DAS = 0 days after submergence, 7 DAS = 7 days after submergence, and 7 DAR = 7 days after recovery	Shoot length (cm)	0 DAS	ns	ns	ns
		7 DAS	***	***	***
		7 DAR	***	***	***
	SPAD value	0 DAS	ns	ns	ns
		7 DAS	***	***	***
		7 DAR	***	***	***
	Maximum quantum yield (FvFm ⁻¹)	0 DAS	ns	ns	ns
		7 DAS	***	***	***
		7 DAR	***	***	***

Fig. 1 Shoot length of IR64 and NIL-SUB1DRO1. Different letters in the same column indicate a significant difference at $p < 0.05$ according to the least significant difference (LSD) test. Error bars represent the standard deviation

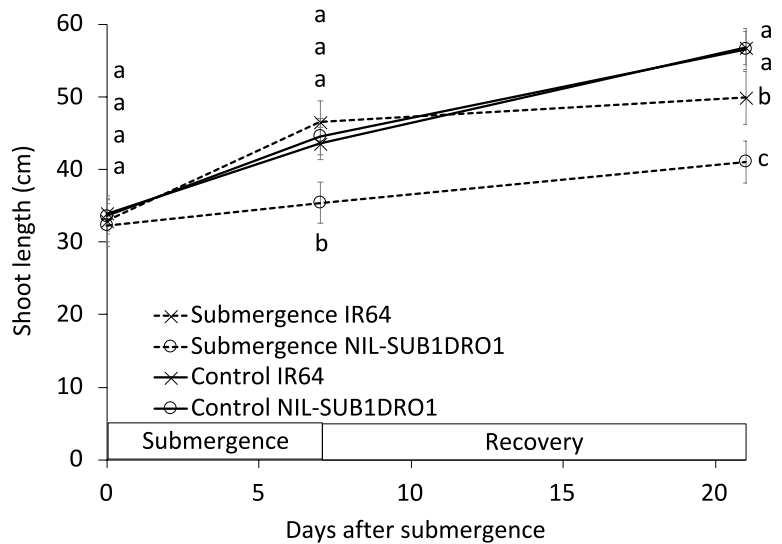
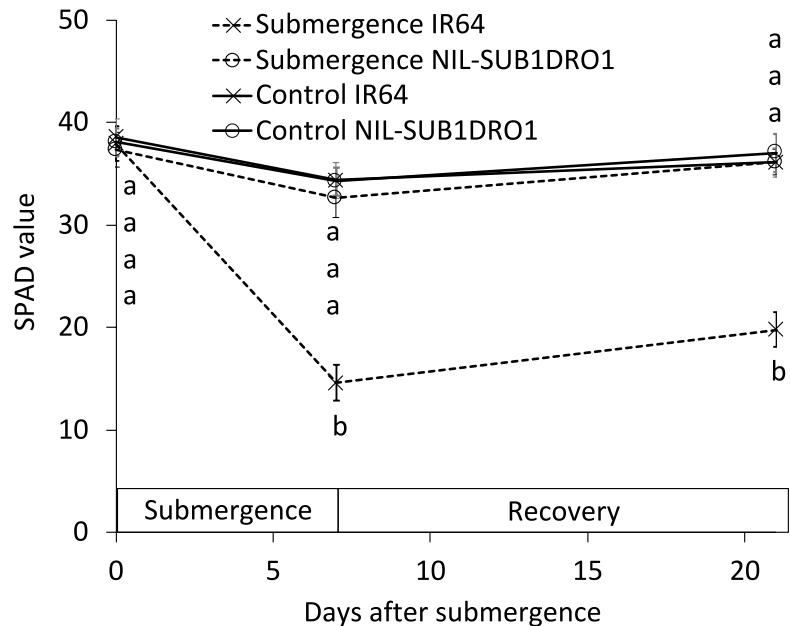


Fig. 2 SPAD value of IR64 and NIL-SUB1DRO1. Different letters in the same column indicate a significant difference at $p < 0.05$ according to the least significant difference (LSD) test. Error bars represent the standard deviation



Although there was no significant difference between control plants and submerged NIL-SUB1DRO1 in SPAD readings after seven days of recovery from submergence, the SPAD values for NIL-SUB1DRO1 were significantly higher than those for submerged IR64. Additionally, chlorophyll fluorescence (F_v/F_m) did not differ significantly between control plants and submerged NIL-SUB1DRO1, while submerged IR64 exhibited significantly lower F_v/F_m (0.63) compared to the control plants (Fig. 3).

Effect of drought on rice growth condition

Experiment 2-1

The soil water content at the start of the drying treatment, when irrigation was stopped, averaged $0.189 \text{ (m}^3/\text{m}^3\text{)}$. On the other hand, the soil water content on the last day of the drought treatment, 29 days after the stop of irrigation, decreased to an average of $0.069 \text{ (m}^3/\text{m}^3\text{)}$. Twenty-nine days of drought significantly

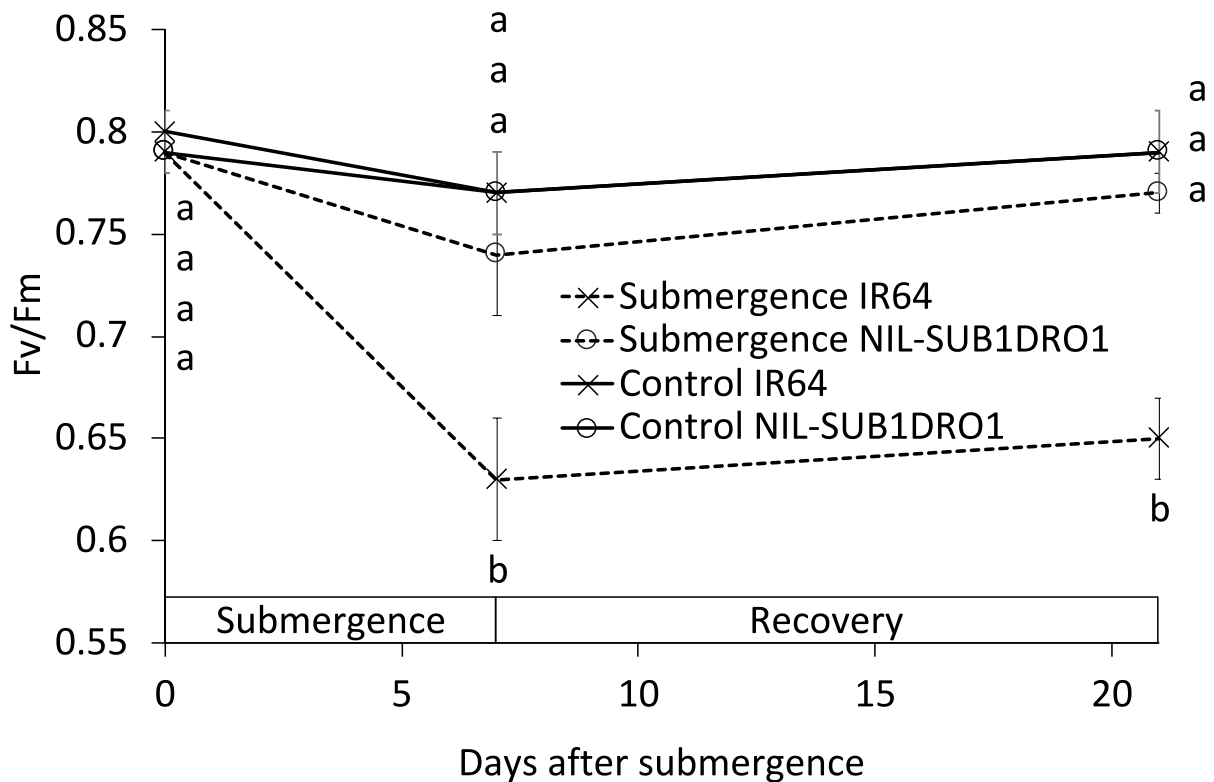


Fig. 3 Maximum quantum yield (Fv/Fm) of IR64 and NIL-SUB1DRO1. Different letters in the same column indicate a significant difference at $p < 0.05$ according to the least significant difference (LSD) test. Error bars represent the standard deviation

reduced the tiller number and leaf area of IR64, NIL-DRO1, and NIL-SUB1DRO1 (Fig. 4 and 5). The leaf area of IR64, NIL-DRO1, and NIL-SUB1DRO1 decreased by 82.6%, 62.25, and 72.05% respectively, while their tiller number decreased by 42.8%, 25.0%, and 29.4% respectively compared to control plants. IR64 was significantly lower than NIL-DRO1 and NIL-SUB1DRO1 and no significant between NIL-DRO1 and NIL-SUB1DRO1 for tiller number under drought condition. The leaf area of drought IR64 was lower than drought NIL-SUB1DRO1 and significantly lower drought NIL-DRO1 after 29 days of drought condition.

Experiment 2-2

The soil moisture status of IR64 and NIL-SUB1DRO1 is presented in Fig. 6. Under control conditions, there was no change in soil moisture content for both genotypes. However, on the second day after ceasing irrigation (drought treatment), the soil

moisture content of both IR64 and NIL-SUB1DRO1 began to decrease. By the end of the 18-day drought treatment, it had decreased by 53.9% for IR64 and 53.5% for NIL-SUB1DRO1 compared to control pipes. During the recovery period, soil moisture levels increased similarly for both genotypes.

The morpho-physiological performance of both genotypes was significantly influenced by the interaction of genotype and environment (Table 3). Notably, there was a significant difference in shoot length between IR64 and NIL-SUB1DRO1 after the drought and recovery periods (Fig. 7). While IR64's shoot length increased by 19.6%, NIL-SUB1DRO1 exhibited a more substantial increase of 44.3% during the drought period compared to start of drought treatment. Drought stress led to decreased chlorophyll content (SPAD value) in both NIL-SUB1DRO1 and IR64, with reductions of 8.1% and 26.2%, respectively, after 18 days of drought compared to the control. Interestingly, NIL-SUB1DRO1 maintained significantly higher chlorophyll content after recovery

Fig. 4 Tiller number of IR64, NIL-DRO1, and NIL-SUB1DRO1. Different lowercase letters indicate a significant difference at $p < 0.05$ according to the least significant difference (LSD) test. Error bars represent the standard deviation

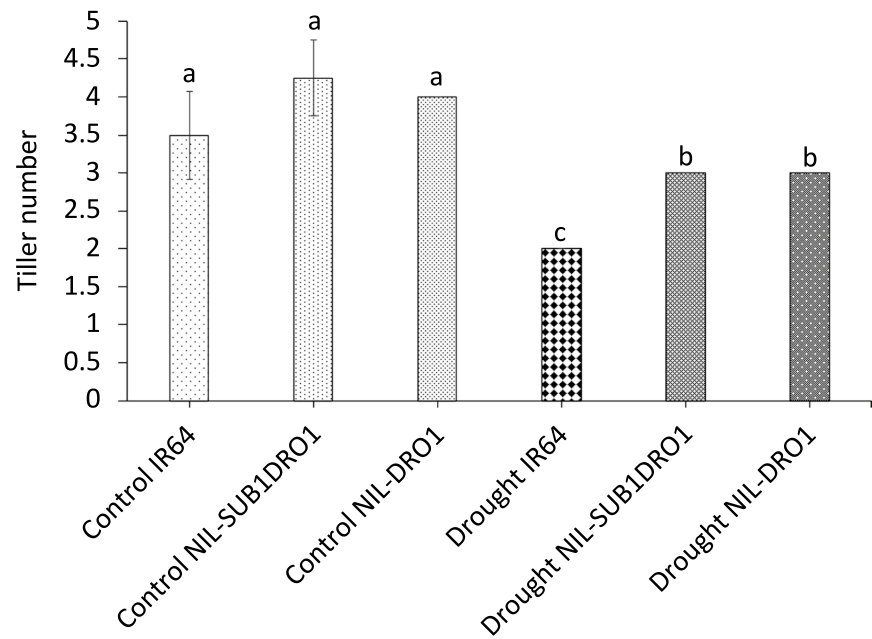
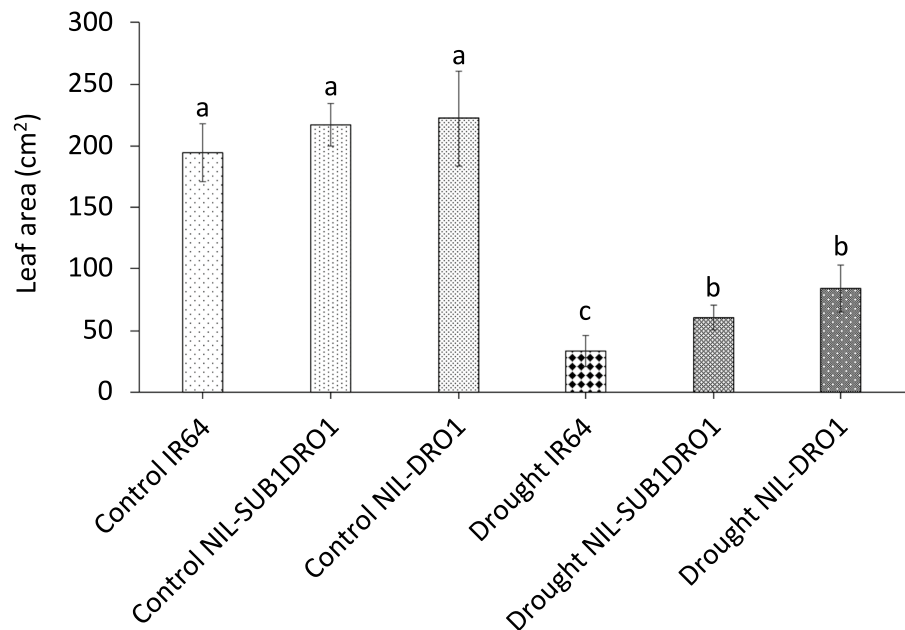


Fig. 5 Leaf area of IR64, NIL-DRO1, and NIL-SUB1DRO1. Different letters indicate a significant difference at $p < 0.05$ according to the least significant difference (LSD) test. Error bars represent the standard deviation



from drought than IR64 (Fig. 8). Stomata play a crucial role in drought tolerance by controlling CO₂ uptake and transpiration rates. To explore stomatal responses under drought stress and during the recovery period, stomatal conductance (gs) was measured (Fig. 9). Although NIL-SUB1DRO1 exhibited slightly lower stomatal conductance than IR64 during

the drought period, this difference was not significant. Furthermore, there was no significant difference in stomatal conductance between the two genotypes during the recovery stage or under control conditions, suggesting that neither of the two genes significantly influenced stomatal responses.

Fig. 6 Soil moisture content in the pipes over days after sowing in Experiment 2-2

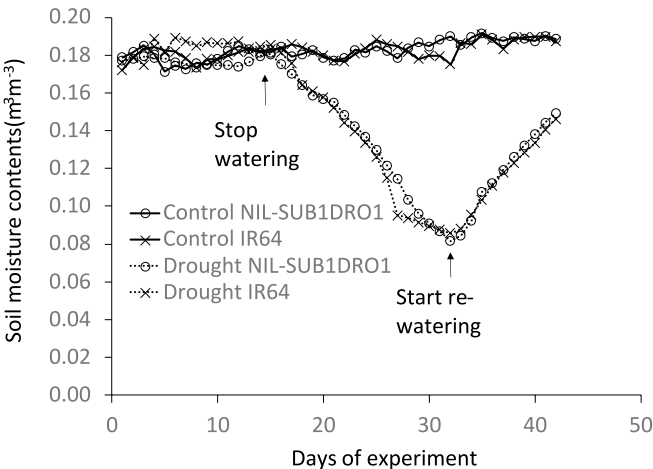


Table 3 Analysis of variance (ANOVA) for the drought Experiment 2-2

Parameter	Collection date	Variety	Environment	Interaction (G X E)
Shoot length (cm)	0 DAD	ns	ns	ns
	18 DAD	***	***	***
	10 DAR	***	***	***
SPAD value	0 DAD	ns	ns	ns
	18 DAD	***	***	***
	10 DAR	***	***	**
Stomata conductance (mmolm ⁻² s ⁻¹)	0 DAD	ns	ns	ns
	18 DAD	***	***	**
	10 DAR	ns	ns	ns
Leaf area (cm ²)	0 DAD	ns	ns	ns
	18 DAD	***	***	***
	10 DAR	***	***	***
Leaf relative water content (%)	0 DAD	ns	ns	ns
	18 DAD	***	***	***
	10 DAR	***	**	*
Leaf water potential (MPa)	0 DAD	ns	ns	ns
	18 DAD	***	***	***
	10 DAR	***	***	***
Shoot dry weight (mgplant ⁻¹)	0 DAD	ns	ns	ns
	18 DAD	***	**	**
	10 DAR	***	***	***
Root dry weight (mgplant ⁻¹)	0 DAD	ns	ns	ns
	18 DAD	***	*	*
	10 DAR	***	**	ns
Root length (cm)	18 DAD	***	***	***
	10 DAR	***	***	***
Root surface area (cm ²)	18 DAD	***	***	**
	10 DAR	***	***	**
Root volume (cm ³)	18 DAD	***	***	***
	10 DAR	***	***	***

*Significant at $p < 0.05$,
**significant at $p < 0.01$,
***significant at $p < 0.001$,
and ns = not significant.
0 DAD = 0 days after drought, 18 DAD = 18 days after drought, and 10 DAR = 10 days after recovery

Fig. 7 Shoot length of IR64 and NIL-SUB1DRO1. Different letters in the same column indicate a significant difference at $p < 0.05$ according to the least significant difference (LSD) test. Error bars represent the standard deviation

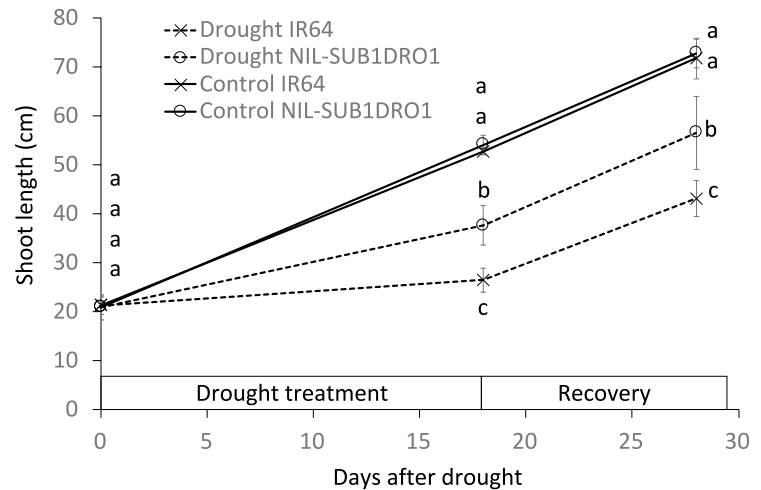
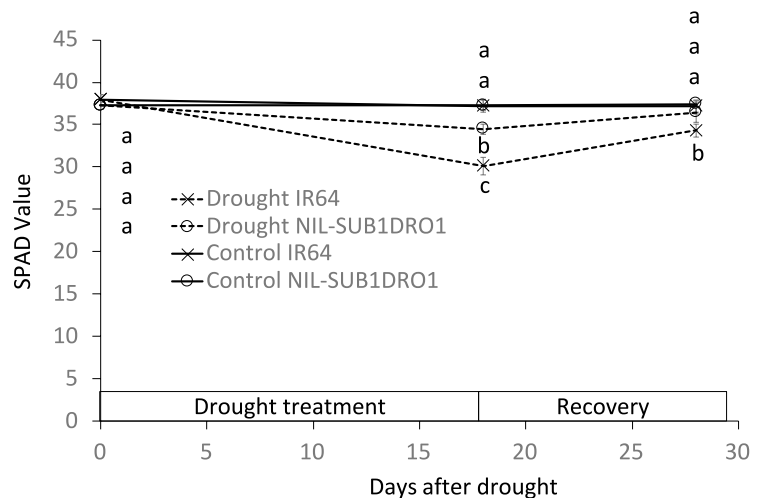


Fig. 8 SPAD value of IR64 and NIL-SUB1DRO1. Different letters in the same column indicate a significant difference at $p < 0.05$ according to the least significant difference (LSD) test. Error bars represent the standard deviation



Drought stress also impacted the leaf area (Fig. 10) in Experiment 2-2. IR64 had a significantly lower leaf area compared to NIL-SUB1DRO1 after both the drought and recovery periods. The mean leaf relative water content and leaf water potential of IR64 were significantly reduced under drought conditions compared to the control. Specifically, IR64's relative water content decreased by 9.3% during the drought, while NIL-SUB1DRO1 only experienced a 2.7% reduction compared to control plants (which was not significant) (Fig. 11). Interestingly, IR64 did not recover well after 10 days of recovery in terms of leaf relative water content (Fig. 11). Additionally, IR64 exhibited significantly lower leaf water potential after drought (-3.88 MPa) and recovery (-2.22 MPa)

compared to NIL-SUB1DRO1 (-2.34 MPa after drought and -1.83 MPa after recovery) (Fig. 12). Finally, drought stress significantly reduced shoot DW for both NIL-SUB1DRO1 and IR64 (by 41.9% and 53.2%, respectively) and root DW for IR64 (by 65.6%) and NIL-SUB1DRO1 (by 40.0%) compared to control plants (Fig. 13a and b).

The ratio (B-layer/A + B-layers) of root morphological characteristics, including total root length, root surface area, and root volume, after drought and recovery is presented in Table 4. Significantly higher ratios (B-layer/A + B-layers) were observed for NIL-SUB1DRO1 compared to IR64 after both the drought period and recovery from the drought. However, no significant differences were found between the two

Fig. 9 Stomatal conductance of IR64 and NIL-SUB1DRO1. Different letters in the same column indicate a significant difference at $p < 0.05$ according to the least significant difference (LSD) test. Error bars represent the standard deviation

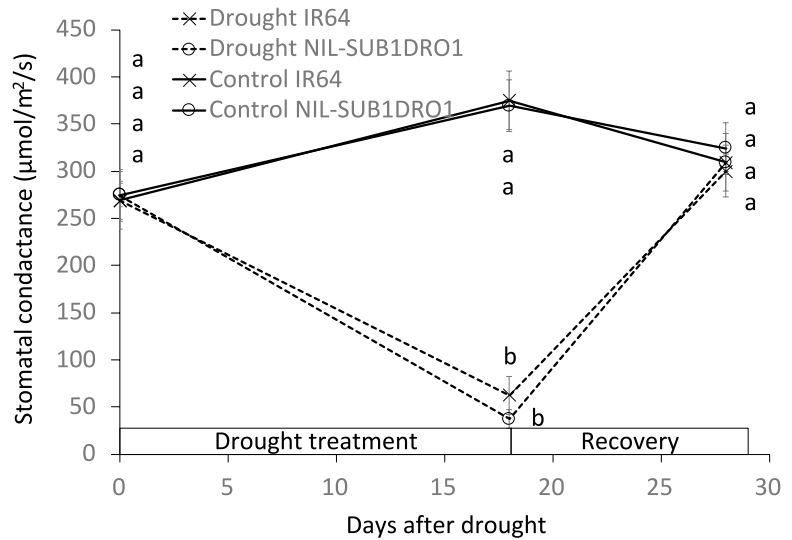
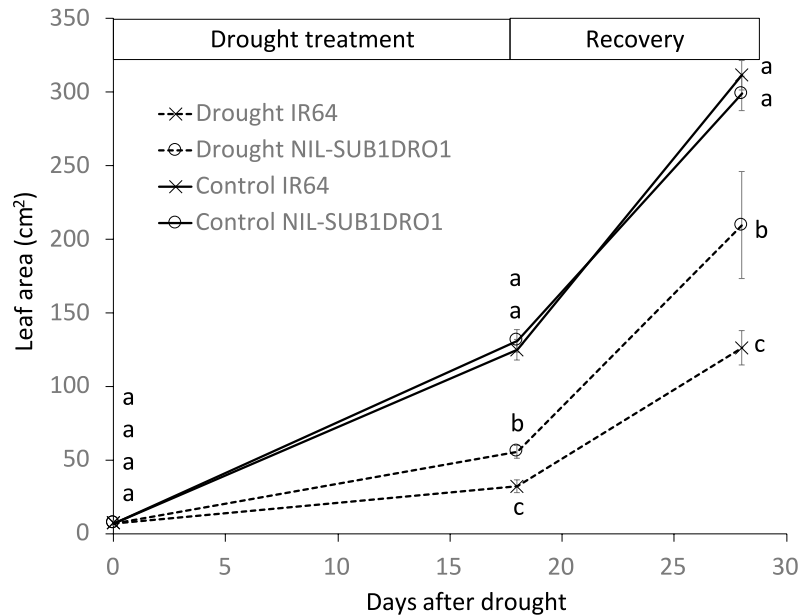


Fig. 10 Leaf area of IR64 and NIL-SUB1DRO1. Different letters in the same column indicate a significant difference at $p < 0.05$ according to the least significant difference (LSD) test. Error bars represent the standard deviation



genotypes under control conditions for these root morphological characteristics.

Discussion

Responses of rice plant to complete submergence condition

The significant effect of submergence and genotype interaction was the increase in shoot length observed

in IR64 compared to NIL-SUB1DRO1 under submerged conditions (Fig. 1) in Experiment 1-2. According to the mechanism of submergence defined by Nagai et al. (2010) and Hattori et al. (2011), IR64's elongation represents transient submergence intolerance, while NIL-SUB1DRO1's relative quiescence reflects tolerance mediated by *SUB1A*. Shoot elongation during submergence, caused by flash flooding, can have adverse effects on survival due to wasted carbohydrates and lodging after desubmergence (Ram et al. 2002). NIL-SUB1DRO1,

Fig. 11 Leaf relative water content of IR64 and NIL-SUB1DRO1. Different letters in the same column indicate a significant difference at $p < 0.05$ according to the least significant difference (LSD) test. Error bars represent the standard deviation

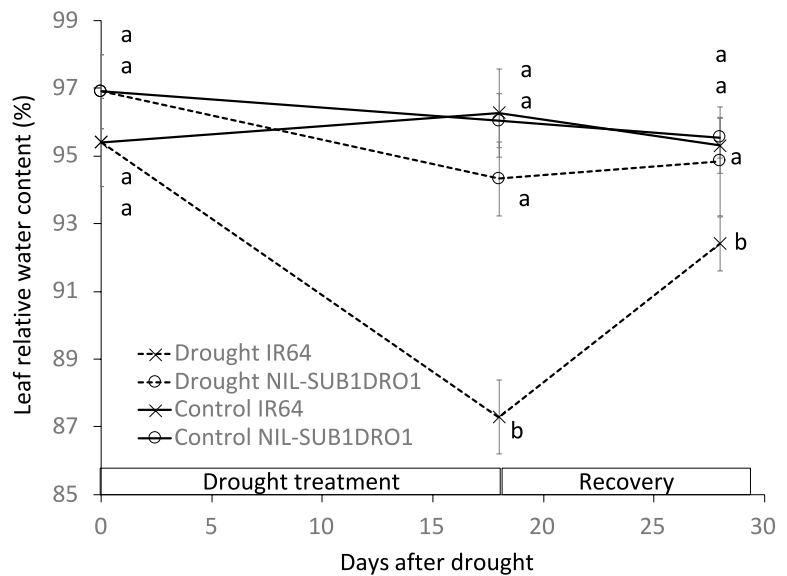
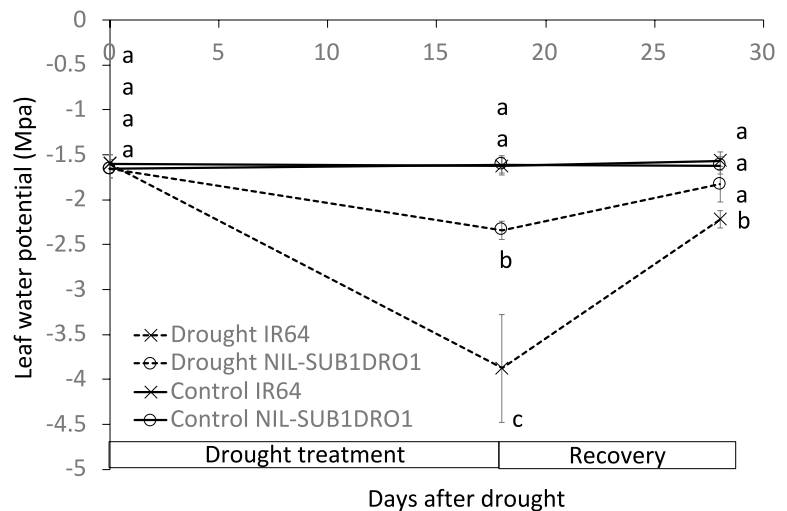


Fig. 12 Leaf water potential of IR64 and NIL-SUB1DRO1. Different letters in the same column indicate a significant difference at $p < 0.05$ according to the least significant difference (LSD) test. Error bars represent the standard deviation



carrying the *SUB1A* gene, limits plant elongation during submergence (Nurrahma et al. 2021a). Chlorophyll fluorescence (F_v/F_m) serves as an effective indicator of rice submergence tolerance (Sone et al. 2012). The significant decline in chlorophyll fluorescence observed in submerged IR64 after 10 and 7 days under water likely reflects reduced PSII activity, disorganisation of the photosynthetic apparatus, and decreased light intensity and oxygen levels in floodwater (Panda and Sarkar 2012). Photoinhibition damage occurs in response to environmental stress, leading to decreased solar energy conservation during photosynthesis. While both sensitive and tolerant

genotypes experience reductions in chlorophyll concentrations during submergence, the effects are relatively greater in sensitive genotypes (Ella et al. 2003; Singh et al. 2014). Leaf chlorosis, observed in the sensitive genotype, IR64 under both submergence experiments, further reduces photosynthesis. Additionally, submerged rice may experience reduced photosynthetic rates due to declining light intensity and gas diffusion rates (Nurrahma et al. 2021b). Desougi et al. (2022) emphasised that chlorophyll plays a crucial role in light absorption, transformation, and energy transmission in plants. Therefore, higher chlorophyll content in submerged plants enhances

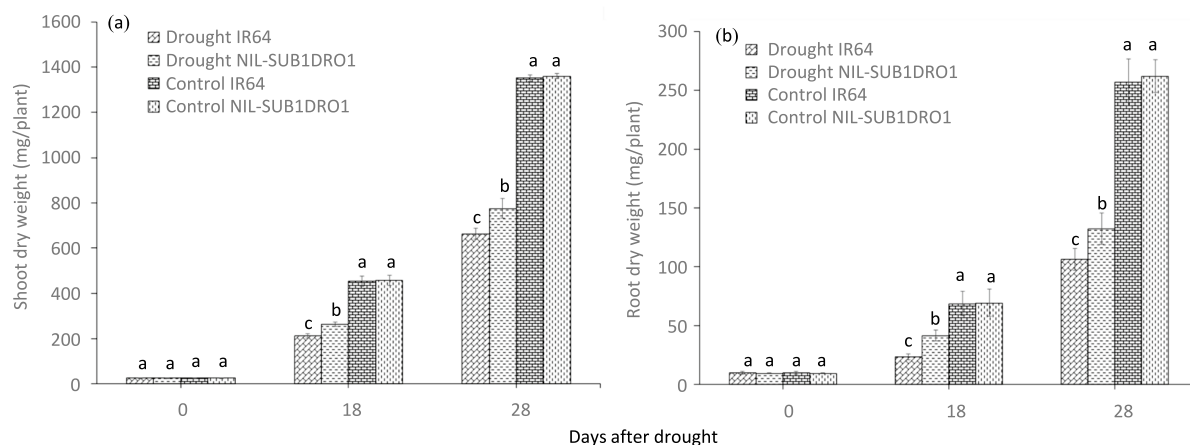


Fig. 13 Shoot (a) and root (b) dry weight of IR64 and NIL-SUB1DRO1. Different letters at the same time of data collection indicate a significant difference at $p < 0.05$ according to

the least significant difference (LSD) test. Error bars represent the standard deviation. 0=0 days after drought, 18=18 days after drought, and 28=10 days after recovery

Table 4 Ratios of root length, surface area and volume after drought and recovery (B-layer/A + B-layers) of Experiment 2-2

***Significant at $p < 0.001$ and ns = not significant. 18 DAD = 18 Days after drought and 10 DAR = 10 Days after recovery

			Root length (cm)		Root surface area (cm ²)		Root volume (cm ³)	
			Control	Drought	Control	Drought	Control	Drought
18 DAD	IR64		0.46	0.21	0.57	0.32	0.46	0.22
	NIL-SUB1DRO1		0.46	0.36	0.58	0.46	0.45	0.38
	T-test		ns	***	ns	***	ns	***
10 DAR	IR64		0.68	0.35	0.41	0.26	0.52	0.37
	NIL-SUB1DRO1		0.67	0.44	0.41	0.33	0.51	0.45
	T-test		ns	***	ns	***	ns	***

carbohydrate production and survival chances. Evaluating chlorophyll content can serve as an indicator of potential dry matter production and plant survival (Singh et al. 2014).

Responses of rice plant to drought condition

The recent development of high-density linkage maps has provided tools for dissecting the genetic basis of complex traits like drought tolerance into individual components. These efforts have led to the identification of quantitative trait loci (QTLs) related to drought tolerance components such as osmotic adjustment (Robin et al. 2003), stomatal regulation (Price et al. 1997), leaf water status and root morphology (Kamoshita et al. 2002). The ability of a plant to recover after drought stress is also crucial. Some researchers suggest that drought recovery ability is

more important than drought tolerance (Sarkar and De Datta 1975). In this study, genotype and environment interactions significantly affected the performance of IR64, NIL-DRO1, and NIL-SUB1DRO1 after 29 days of drought and NIL-DRO1 and NIL-SUB1DRO1 after 18 days of drought and 10 days of recovery, except for root DW and stomatal conductance after recovery. During the drought and recovery periods, IR64's leaf relative water content, leaf water potential, leaf area, and SPAD value were significantly lower than in control plants. According to Fofana et al. (2018), the significantly higher leaf area of NIL-SUB1DRO1 and NIL-DRO1 in this study after drought stress could be supported by the leaf's morphology (flatter leaves and fewer dead leaves), suggesting a higher assimilatory surface for light capture and transpiration. The higher stomatal conductance observed in IR64 over NIL-SUB1DRO1 though

not significant during the drought period could have predisposed the variety to a change in water balance through increased water loss under reduced supply, Xingyun et al. (2023) reported similar findings. Under water-limited environments, a plant's initial response is to prevent a decline in water content by balancing water uptake and water loss rates, a stress avoidance strategy (Verslues et al. 2006). Stomatal closure is the immediate and short-term mechanism plants employ in response to potential water loss (Oliver et al. 2010). Stomatal conductance is a component of total diffusion that involves mesophyll diffusion. Serraj et al. (2008) suggested that rice is better described as a dehydration avoider; thus, the higher stomatal conductance observed for IR64 could be a disadvantage for drought tolerance. Soil moisture stress affects plants' water status through water potential components (Chakraborty et al. 2008). Farooq et al. (2012) observed that leaf hydraulic conductance affects responses to drought stress. Reduced cellular turgidity implied by reduced water potential disrupts the structural integrity of leaf cells, causing electrolyte leakage (Farooq et al. 2012). Demirevska et al. (2008) noted a significant reduction in leaf relative water content with increasing drought severity. Disruption in water balance and changes in leaf morphology negatively affect other physiological processes, such as transpiration and photosynthesis (Fofana et al. 2018). This partly explains the significantly lower shoot DW of IR64 plants under soil moisture stress (Fig. 13a). *DRO1* likely regulates stomatal function under dry conditions to prevent moisture loss, in addition to enhancing water uptake.

Rice is a shallow-rooted crop susceptible to drought. Root growth becomes restricted during exposure to abiotic stresses, including drought (Kato and Okami 2010). To adapt to water-limited conditions, rice roots undergo morphological and anatomical changes (Kato and Katsura 2014). The ability to develop the root system in response to drought stress reflects phenotypic plasticity (Dien et al. 2017). According to Yoshida and Hasegawa (1982), since plants acquire water from the soil, root growth is crucial for drought stress resistance. O'Toole and Chang (1979) found that rice varieties with longer and thicker roots were more drought-tolerant than those with shorter and thinner roots. In this study, the ratio of deeper to shallower roots (B-layer/A + B-layers) for NIL-SUB1DRO1 was significantly higher than for

IR64 after drought and recovery for total root length, root surface area, and root volume (Table 4). The study's results align with previous reports, indicating that larger root systems (deeper roots, higher root surface area, and root volume) are important for drought tolerance in rice (Dien et al. 2017). The *DRO1* gene enhances the development and distribution of deeper roots under relatively dry upland conditions to acquire water and other mineral elements essential for plant growth (Uga et al. 2011).

Effect of combined submergence and drought tolerance genes

Apparently, introgressing both *SUB1* and *DRO1* through breeding will likely enhance rice adaptation in areas affected by both flooding and drought, either within the same season or during different years, as is common in most rainfed lowlands due to changing climatic conditions. Combining the two genes does not seem to have negative interactions and is likely expressed independently. Further studies are needed to verify these results in natural farmers' fields.

Conclusions

Submergence and drought are serious abiotic stresses affecting rice survival and growth, significantly impacting high-yielding varieties like IR64. NIL-SUB1DRO1 combines the submergence tolerance gene and the deep-rooting gene in the genetic background of IR64, while NIL-SUB1 and NIL-DRO1 contains submergence tolerance and deep-rooting genes respectively in the genetic background of IR64 as well. NIL-SUB1DRO1 exhibited characteristics associated with submergence stress tolerance, reflected in the quiescence strategy that suppresses the elongation of the above-ground part during flooding and maintains a higher Fv/Fm. NIL-SUB1 also maintained a higher Fv/Fm value during submergence. When subjected to dry soil conditions, NIL-SUB1DRO1 displayed favourable deep-rooting properties, including improved root length, root surface area, and root volume in the lower soil layers, NIL-DRO1 also maintained stable tiller number and leaf area. Combining the two genes does not have any apparent negative impacts under either stress condition. The significant interaction between genotype

and environment in both experiments demonstrates that these genotypes have a genetic effect in different environments. Future research should confirm the effect on tolerance and resilience conferred by these two genes during early growth stages, adaptation, and ultimately grain yield under natural field conditions.

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Author contributions J.I. S. designed the study; I.S. and N.T.T. H. conducted the study; T.O. provided rice genotypes NIL-SUB1, NIL-DRO1 and NIL-SUB1DRO1; E.O. and R.C. assisted in data collection and analysis. I.S., A.M.I. and J.I.S. wrote the original draft. All authors participated in review and editing of the manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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