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Research Article

The Effects of Submergence on Selected Malaysian Rice Varieties

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ABSTRACT

Various varieties have been developed in Malaysia, mainly to improve rice response to environmental changes, pests, and diseases, as well as to increase rice productivity under stressful conditions. Despite being semi-aquatic plants, rice is intolerant to complete submergence for a long period. This study was conducted to evaluate the response of seven Malaysian rice varieties at the vegetative stage under submergence stress. Two-week-old rice seedlings were submerged for 14 days, and the changes in plant height, chlorophyll content, and soluble sugar content were determined. The survival percentage of these varieties was observed after 14 days of de-submergence, where UKMRC2 and MR220CL possessed high survivability (90% & 60%, respectively). After submergence, all varieties showed height increment and reduced chlorophyll and soluble sugar contents. Based on our analyses, UKMRC2 performed better than other varieties, although slightly less than IR64-Sub1. It was confirmed that UKMRC2 is the submergence-tolerant variety, and its response to underwater germination was also determined. Our result showed that UKMRC2 might possess tolerance to anaerobic germination conditions, and more studies are needed to understand its molecular mechanism for submergence. In conclusion, many varieties used were susceptible to submergence, and the development of more submergence-tolerant varieties is crucial for Malaysia's food security sustainability.

Key words: Modern rice varieties, quiescence strategy, submergence, UKMRC2, underwater germination

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INTRODUCTION

Unpredictable changes in temperature, rainfall, and weather patterns are the environmental factors that influence the growth, productivity, and survival of crop plants. Flooding is one of the abiotic stresses that negatively affect rice production worldwide, especially in Southeast Asia, and has become a devastating issue for farmers. In Malaysia, flooding destroyed about 1,500 hectares of rice planting area, especially in Kedah, Kelantan, and Pahang (DOA, 2015), and caused a huge impact on national rice production and the socio-economic status of local farmers.

Rice is a semi-aquatic plant, which can be partially or completely submerged during flooding. The response of rice to flooding or submergence depends on the rice genotypes, the intensity, and duration of rainfall, and the characteristics of floodwater and topography (Ito *et al.*, 1999; Meng *et al.*, 2022). The increase in water level during flooding disrupts oxygen (O_2) and carbon dioxide (CO_2) diffusions into rice plants, interferes with the electron flow during photosynthesis and respiration, increases the energy consumption, and limits the production of sugar for the plant's growth (Jackson & Ismail, 2015). Under prolonged submergence, plants may suffer nutrient deficiency and energy starvation, leading to fatality (Ito *et al.*, 1999; Tamang & Fukao, 2015).

Despite the negative impact of flooding, certain rice genotypes may thrive under submergence conditions by adapting several strategies to avoid or escape from this type of stress (Afrin *et al*., 2018). Several anatomical and morphological features are beneficial for rice adaptations to submergence, such as the formation of aerenchyma tissues, leaf gas film, and adventitious roots, which can facilitate the translocation of oxygen to submerged rice tissues (Ito *et al*., 1999; Kuroha & Ashikari, 2020; Panda & Barik, 2021). During the germination stage, anaerobic germination (AG) under submergence causes rapid coleoptile elongation and delayed radicle development in the submergence-tolerant rice, as starch reserves are utilized through higher amylase activity and anaerobic respiration (Ismail *et al*., 2009). At the vegetative stage, the growth of seedlings can be either suppressed until the level of water is reduced (quiescence strategy) or the leaf internode is rapidly elongated to reach out to the water surface (escape strategy) (Hattori *et al*., 2009; Bailey-Serres *et al*., 2010; Kuroha & Ashikari, 2020).

The quiescence strategy is adopted by submergence-tolerant rice, where shoot elongation is restrained to reduce carbohydrate consumption during submergence, and energy is conserved to recommence plant growth and development after the submergence condition ends (Das *et al*., 2005; Tamang & Fukao, 2015). This adaptation is mediated by SUBMERGENCE 1A (SUB1A), a protein that belongs to the ETHYLENE RESPONSE FACTOR (ERF) superfamily (Xu *et al*., 2006). SUB1A influences carbohydrate metabolism, low energy sensing pathways, and sugar homeostasis under submergence stress, as well as maintaining photosynthetic capacity and promoting plant recovery post-submergence (Locke *et al*., 2018; Perata, 2018). Rapid internode elongation is the escape strategy adapted by deepwater rice exposed to a long period of flooding (Nishiuchi *et al*., 2012). SNORKEL1 (SK1) and SK2 are the members of the ERF family (Hattori *et al*., 2009) that induce the activation of ethylene biosynthesis, which further increases the accumulation of gibberellic acid (GA) and represses abscisic acid (ABA) production (Kende *et al*., 1998; Hattori *et al*., 2011).

Many varieties have been developed in Malaysia to obtain high-yielding rice and be tolerant to environmental stresses. MR219, MR284, and MR297 are high-yield varieties that are resistant to rice blast disease (MADA, 2017), whereas UKMPL-5 and UKMRC8 are tolerant to drought and submergence, respectively (Shamsuddin *et al*., 2016; Ikmal *et al*., 2019). Depending on the granary area, several varieties are preferred by farmers for their short maturity and tolerance to disease and pests. For example, MR303 and MR220CL varieties were planted in Bachok, Kelantan (KADA, 2021) because of their high yield, resilience to the unfavorable environment, and tolerance to herbicides (Sunian *et al*., 2019). However, the responses of these varieties to submergence have yet to be determined. Bachok is one of the regions that was affected by floods during the monsoon (Razali, 2019). Thus, the planting of submergence-tolerant rice among local farmers is highly needed.

To date, except for MR303 and MR220CL, the response of several local varieties to submergence stress has been evaluated, and most of these varieties are susceptible to flooding (Ikmal *et al*., 2019; Sazali *et al*., 2021). The present study was conducted to evaluate the morpho-physiological changes of MR303 and MR220CL as well as other varieties under submergence at the vegetative stage. In addition, the anaerobic germination (AG) response of the submergence-tolerant variety, UKMRC2, was also carried out to investigate its tolerance level during underwater germination.

MATERIALS AND METHODS

Plant materials and growth condition

Seeds of rice varieties MR219, MR284, MR297, MR303, and MR220CL were obtained from the Kemubu Agricultural Development Authority (KADA), whereas seeds of UKMRC2, UKMPL-5, and IR64-Sub1 were provided by the Universiti Kebangsaan Malaysia (UKM). The IR64-Sub1 was used as a check variety tolerant to submergence stress following a previous study by Septiningsih *et al*. (2009), whereas MR219 was a control for submergence-susceptible variety (Ahmed *et al*., 2016; Ikmal *et al*., 2019; Ahmad *et al*., 2020).

The varieties used in this study were arranged in a randomized complete block design with three replications. The experiments were conducted at the Plant Biotechnology Center and Terrace Q, Rumah Tumbuhan, UKM Bangi, Selangor. Plants were grown in a greenhouse with maximum and minimum temperatures of between 25 °C to 35 °C, respectively. The relative humidity was about 85% throughout the experiment.

Submergence treatment on rice seedlings

All seeds were soaked in a flask containing distilled water (in darkness at 25 °C) for two days' duration. Those seeds were then sowed on a tray containing mixed soils (topsoil: organic matter: sand with a ratio of 3:2:1) under glasshouse conditions for 14 days duration. Next, 30 seedlings with similar heights for each variety were kept on in the tray, and placed in a water tank with a dimension of 1.10 m × 1.10 m × 1.10 m filled with a 1-meter depth water level for 14 consecutive days. Then, all seedlings were taken out from the tank and placed under greenhouse conditions to allow for recovery after submergence. During the recovery phase, plants were watered until they reached maturity.

Plant height and survival percentage

The plant height was measured before (T0) and after 14 days (T14) of submergence. The height was measured from the stem base to the tip of the leaf. The leaves were drooping due to the submergence treatment; therefore, the height was measured one day after (on the 15th day) the plants were taken out of the tank.

Five seeds with four replications were used to determine the survival percentage. The formation of the new green shoot was used to indicate the recovery of the rice. The survival percentage of each variety was determined after 14 days of de-submergence according to the equation below.

$$\text{Survival percentage (\%)} = \frac{\text{Number of survived seedlings}}{\text{Total number of seedlings}} \times 100$$

Chlorophyll content analysis

The chlorophyll content before and after submergence was determined using methods by Arnon (1949). About 100 mg of the leaf was grounded with 80% acetone using a pestle and mortar. The homogenate was then centrifuged at 5,000 ×*g* for 10 min at 4 °C and the supernatant was transferred into a new tube. The supernatant was measured using a spectrophotometer at the absorbance wavelength of 645 nm (A_{645}) for chlorophyll a and 663 nm (A_{663}) for chlorophyll b. The total chlorophyll content was determined using the formula:

$$\text{Total chlorophyll} \left(\frac{mg}{g}\right) = 20.31(A645) + 8.05(A663)$$

Soluble sugar content analysis

The soluble sugar content before and after submergence treatment of each variety was determined following methods by Yoshida *et al*. (1976), The leaf was pat-dried, and about 100 mg of the sample was grounded in liquid nitrogen using a mortar and pestle. The fine powder was transferred into a new tube containing 80% ethanol and subjected to incubation at an 80 °C water bath for 30 min. Then, the sample was centrifuged at 8,000 ×*g* for 3 min at 4 °C, and the supernatant was transferred into a new tube. Next, 95% ethanol (v/v) was added into the tube and placed in the water bath set at 80 °C for 5 min. The sugar extract was then transferred into a conical flask before being added to distilled water. About 5 mL of diluted sugar extract was transferred into a test tube and placed on ice before the addition of the anthrone reagent. The mixture was added slowly using a glass rod and boiled in the water bath for 8 min before letting it cool on ice. The absorbance of the sample at the wavelength of 630 nm was determined using the spectrophotometer, and D-glucose solutions were used as standards.

In all parameters measured under submergence treatment, at least three individual plants were used. The percentage of changes was calculated based on the following equation:

$$\text{Changes in percentage (\%)} = \frac{\text{(Final measurement (Day 14)-Initial measurement (Day 0))}}{\text{Initial measurement (Day 0)}} \times 100$$

Underwater germination

Three varieties, namely MR219, UKMRC2, and IR64-Sub1, were chosen for the underwater germination test. These varieties were selected based on their response to submergence at the vegetative stage in this current study. MR219 and IR64-Sub1 were the controls for susceptible and tolerant varieties, respectively. Five seeds of each variety, in duplicates, were soaked in distilled water for 48 h in the dark. Then, the control seeds were sowed on moist filter paper and allowed for germination in the air. The filter paper was wetted with distilled water every day to avoid it drying out. For underwater germination, seeds were placed in a container filled with 5 cm of distilled water and sealed with a lid. Seeds were germinated at 25 °C under 16 h of light and 8 h of darkness. The coleoptile length for control and submergence treatments was measured with a ruler after 7 days.

Statistical analysis

All data obtained were subjected to a two-way analysis of variance (ANOVA), and Tukey's test was used to analyze the differences among varieties using Minitab v21.1. Correlation analysis between parameters measured under submergence treatment was conducted using GraphPad Prism v9.

RESULTS AND DISCUSSION

Survival percentage of rice under submergence

Submergence treatment was imposed on rice varieties for 14 days, and their survival was determined after 14 days of de-submergence. The check variety, IR64-Sub1, recorded a 100% survival percentage (Figure 1). Among other varieties, UKMRC2 recorded the highest survival percentage at 90%, followed by MR220CL (60%), while the lowest was recorded in MR297 and UKMPL-5 at 10%, respectively. This result suggests that UKMRC2 was highly tolerant to submergence, whereas MR220CL might be partially tolerant to submergence. Our result was corroborated by Ikmal *et al*. (2019), which reported that the survival percentage of UKMRC2 under submergence stress was 67.2%. The UKMRC2 was produced by a cross between MR219 and *Oryza rufipogon* Griff. (IRGC105491) (Wickneswari & Bhuiyan, 2014), does not have the Sub1 QTL (Ikmal *et al*., 2019), thus suggesting that other genes may contribute to UKMRC2's tolerance to submergence. On

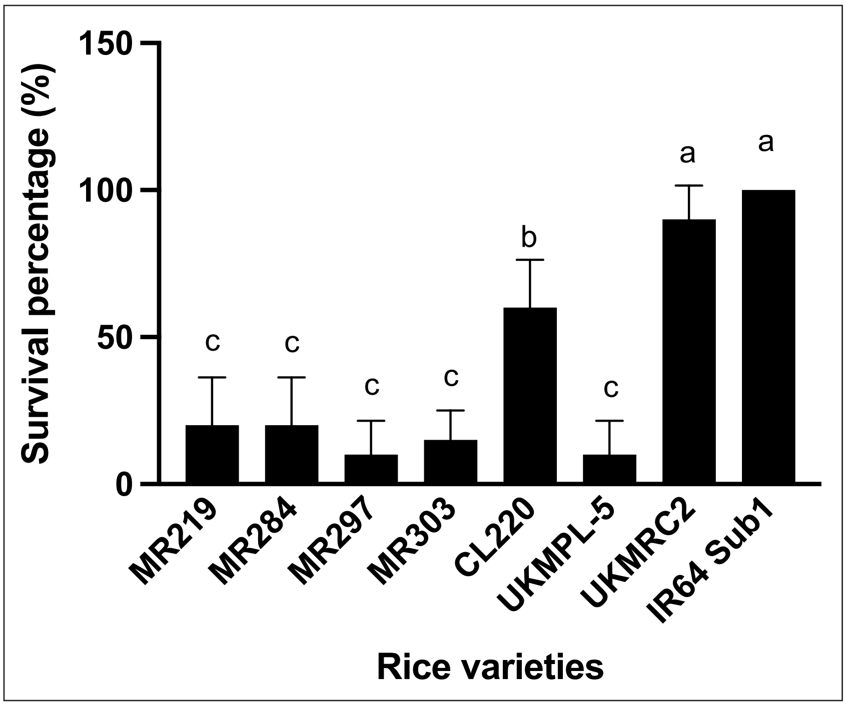

Fig. 1. The survival percentage of rice varieties after 14 days of de-submergence. Means with different letters are significantly different at *p*<0.05.

the other hand, Sazali *et al*. (2021) reported that MR284 and MR297 recorded low survival percentages under saline submergence, which indicated that these varieties were susceptible to submergence conditions.

Morpho-physiological changes of rice varieties under submergence treatment

Plant height

In general, all rice varieties showed an increment in plant height after submergence (Table 1). The highest plant height increment was observed in MR303, whereas IR64-Sub1 was the shortest plant after the submergence treatment ended. Among these varieties, MR303 showed the highest elongation change percentage at 54.9%, followed by MR220CL (27.8%), whereas the lowest increment in plant height was in IR64-Sub1 (11.2%), followed by UKMRC2 (16.8%) (Table 1). This result suggests that the leaf elongation of MR303 was greatly influenced by submergence.

The rice leaf is elongated during submergence, mainly to allow gaseous exchange above the water surface and to ensure the plant can resume respiration and photosynthesis activities (Hattori *et al*., 2009; Bailey-Serres *et al*., 2012; Kuroha & Ashikari, 2020). This adaptation gives an advantage to the plant to escape from the excessive water level and continue to grow. However, cell division and elongation during the extension of the leaf under submergence require energy consumption and carbohydrate depletion, which could cause death if the energy is fully-utilized before the leaf reaches the water surface (Ito *et al*., 1999; Bailey-Serres *et al*., 2010; Sone *et al*., 2012). This may explain the low survival percentage (15%) of MR303 under submergence although it possessed the highest elongation percentage (Table 1).

Changes in plant height were less than 20% for IR64-Sub1, UKMRC2, and MR284, suggesting that energy is conserved in these varieties under submergence. IR64-Sub1 is one of the submergence-tolerant mega varieties (Bailey-Serres *et al*., 2010), and the presence of the Sub1 QTLs is known to enhance tolerance by regulating sugar metabolism (Fukao *et al*., 2006; Locke *et al*., 2018). This quiescence strategy may also be adopted by UKMRC2 in response to submergence, although Sub1 QTLs were absent, as a strategy to avoid exhaustion of energy from internode elongation (Ikmal *et al*., 2019).

Chlorophyll content

Overall, the chlorophyll content of all varieties was significantly reduced after submergence treatment. The highest chlorophyll content was observed in IR64-Sub1, while the lowest was in MR219 (Table 1). In addition, the lowest chlorophyll reduction was observed in IR64-Sub1 (69.9%), followed by UKMRC2 (83.2%) and MR284 (87.2%). Other varieties showed high chlorophyll reduction of more than 90%, with the highest being in MR219 (95.1%).

Table 1. Means and the percentage of changes in plant height, chlorophyll content, and soluble sugar content before and after submergence treatment

Varieties	Plant height (cm)			Chlorophyll content (mg/g)			Soluble sugar content (mg/g)		
	Day 0	Day 14	% of changes	Day 0	Day 14	% of changes	Day 0	Day 14	% of changes
MR219	35.0 ±1.00^{ab}	42.0 ± 2.65^{abc}	20.0	3.98 ± 0.12^{c}	0.20 ± 0.02^{e}	95.1	0.073 ± 0.010^{a}	0.011 ± 0.002^{c}	84.9
MR284	34.3 ± 1.53^{abc}	40.3 ± 1.53^{bc}	17.5	3.35 ± 0.01^{e}	0.43 ± 0.03^{bc}	87.2	0.073 ± 0.008^{a}	0.011 ± 0.001^{c}	84.9
MR297	36.7 ± 0.58^{abc}	45.3 ± 0.58^{bc}	23.6	3.79 ± 0.17^{cd}	0.33 ± 0.04^{cde}	91.2	0.088 ± 0.007^{a}	0.018 ± 0.003^{bc}	79.5
MR303	30.3 ± 0.58^{a}	47.0 ± 2.65^{ab}	54.9	4.91 ± 0.12^{a}	0.38 ± 0.04^{bcd}	92.2	0.073 ± 0.010^{a}	0.029 ± 0.005^{a}	60.3
MR220CL	32.3 ± 0.58^{d}	41.3 ± 2.31^{a}	27.8	3.77 ± 0.02^{cd}	0.25 ± 0.09^{de}	93.5	0.085 ± 0.010^{a}	0.011 ± 0.003^{c}	87.1
UKMPL-5	32.3 ± 0.58^{bcd}	40.0 ± 1.00^{c}	23.7	4.42 ± 0.06^{b}	0.35 ± 0.05^{cd}	92.1	0.073 ± 0.004^{a}	0.015 ± 0.005^{bc}	79.5
UKMRC2	31.7 ± 1.53^{cd}	37.0 ± 1.00^{c}	16.8	3.06 ± 0.03^{f}	0.51 ± 0.04^{b}	83.2	0.073 ± 0.008^{a}	0.010 ± 0.003^{c}	86.3
IR64-Sub1	35.7 ± 0.58^{a}	39.7 ± 0.58^{c}	11.2	3.60 ± 0.14^{de}	1.08 ± 0.04^{a}	69.9	0.084 ± 0.025^{a}	0.025 ± 0.004^{ab}	70.2

Means with different letters for each day are significantly different at $p<0.05$.

Chlorophyll is a green pigment that is essential in perceiving light for photosynthetic activities. Chlorophyll content in plants is greatly influenced by environmental factors such as soil nutrients, temperature, light intensity, and water availability (Du *et al*., 2017; Naznin *et al*., 2019; Ajeng *et al*., 2020; Damm *et al*., 2022), which indirectly affect plant growth and productivity. The reduction of chlorophyll content due to abiotic stresses could be associated with chloroplast degradation through autophagy or chloroplast vesiculation (Jiang *et al*., 2020) and enhanced chlorophyllase activities that promote the degradation of chlorophyll (Sakuraba *et al*., 2014; Sharma *et al*., 2020). The reduced chlorophyll content is expected in rice that was completely submerged due to chlorophyll breakdown and photodamage in leaves, which are mediated by ethylene production (Jackson *et al*., 1987; Sone & Sakagami, 2017). Consequently, chlorosis in submerged leaves inhibits photosynthesis and could lead to plant cell death.

Several rice varieties that have been introgressed with SUB1 showed increased tolerance to submergence compared to the non-introgressed SUB1 rice as they maintained higher chlorophyll content during submergence (Sarkar & Bhattacharjee, 2011; Singh *et al*., 2014; Bui *et al*., 2019). Retention of chlorophyll content is important in ensuring rice survival and recovery after the submergence period ends (Singh *et al*., 2014). In this study, UKMRC2 showed less chlorophyll content reduction than other varieties except for the check variety IR64-Sub1, suggesting that the remaining chlorophyll may facilitate the survival of UKMRC2 during submergence.

Soluble sugar content

Similarly, submergence resulted in a significant reduction of sugar in all varieties (Table 1). The lowest change in sugar content was in MR303 (60.3%), followed by IR64-Sub1 (70.2%) and both MR297 and UKMPL-5 (79.5%). More than 80% sugar content reduction can be seen in other varieties, with the highest reduction being in MR220CL (87.1%) (Table 1).

Submergence-tolerant rice retains the level of non-structural carbohydrates (NSCs: soluble sugar and starch) because it is essential in enhancing the submergence tolerance of rice plants, particularly by maintaining growth parameters (Sarkar, 1997), shoot elongation ability (Sarkar *et al*., 1996; Das *et al*., 2005), and sustaining sucrose metabolism (Panda & Sarkar, 2014). Higher carbohydrate content after submergence is often associated with rice survival, where new leaf regeneration is required for further photosynthetic activities and re-activation of plant growth (Sarkar & Battacharjee, 2011). Energy conservation will encourage recovery and improve chances of survival after de-submergence, in contrast to the escape strategy, which uses more energy during internode elongation (Bui *et al*., 2019).

Soluble sugars such as glucose, fructose, and maltose are needed to regulate various metabolic processes in plants. Soluble sugars can detect environmental changes and function as signals in sugar signaling pathways in response to abiotic stress (Rosa *et al*., 2009). During submergence, increases in soluble sugars could improve cells' osmotic potential to protect the cell from oxidative damage (Lv *et al*., 2016). Soluble sugars are consumed for leaf elongation and are associated with the survivability of rice after de-submergence (Sayani *et al*., 2017). In susceptible varieties, soluble sugars would normally be used up as a strategy to escape from submergence, but the plant cannot survive afterward. Meanwhile, tolerance varieties could have either a low amount of soluble sugars with restricted leaf growth or high soluble sugars, promoting leaf elongation and surviving from submergence (Samanta *et al*., 2021).

In this study, MR303 contained the highest soluble sugar content and the longest plant, but the survival percentage accounted for 15% only. These results suggest that MR303 used up the soluble sugars for its leaf elongation but mostly would not survive submergence. In contrast to IR64-Sub1, high soluble sugars might not be fully utilized for leaf elongation and were conserved for post-submergence growth.

Correlation analysis

Among the parameters examined in this study, chlorophyll content after submergence (CCA) showed a positive correlation with survival percentage (r=0.70, p<0.05), whereas plant height after submergence (PHA) was negatively correlated with survival percentage (r=-0.64, p<0.05). Meanwhile, chlorophyll content before submergence (CCB) was positively correlated (r=0.71, p<0.05) with plant height after submergence (PHA) (Figure 2). These results suggest that chlorophyll content is associated with leaf growth and the survival of seedlings under submergence conditions.

Chlorophyll is a crucial pigment for photosynthesis, which could affect the growth of leaves during submergence stress. In addition, the amount of chlorophyll may facilitate certain genotypes' survival during submergence. The ability to adapt to aerobic conditions after de-submergence is probably due to the high chlorophyll content in the leaves that emerged during submergence through a decreased amount of carbohydrate breakdown (Sone *et al*., 2012). Meanwhile, a negative correlation between plant height and survival has already been established in submergence-susceptible varieties, since it is known that energy deprivation during leaf elongation could lead to plant death (Bailey-Serres *et al*., 2010; Sone *et al*., 2012).

Anaerobic germination of UKMRC2

MR219, UKMRC2, and IR64-Sub1 were selected for underwater germination tests based on their survival percentage during submergence stress. Our results showed that all varieties were fully germinated within 7 days of submergence. The coleoptile of IR64-Sub1 was significantly reduced under submergence, but the coleoptiles of MR219 and UKMRC2 were elongated more than 5 cm during submergence (Figure 3). These results suggested that anaerobic germination caused faster coleoptile elongation in both MR219 and UKMRC2 as compared to IR64-Sub1.

In general, tolerant rice genotypes can germinate and elongate their coleoptile underwater since the carbohydrate reserves in the endosperm are utilized for the establishment of seedling growth (Lee *et al*., 2014). Rice genotypes that are tolerant to anoxic (without oxygen) or hypoxic (limited oxygen) conditions showed higher rates of coleoptile elongation, alcoholic fermentation, and glycolysis-related enzyme activities than the intolerant variety (Gibbs *et al*., 2000; Lee *et al*., 2009). Based on our results, UKMRC2 may be able to tolerate anaerobic germination by extending its coleoptile length underwater, but more analyses are required to confirm this finding.

CONCLUSION

In conclusion, our study indicated that most local rice varieties were susceptible to submergence stress, except for UKMRC2. Hence, more efforts are needed to produce submergence-tolerant rice for local consumption. In addition, further study on the molecular mechanism of UKMRC2 via genetics and molecular approaches is needed to identify genes or QTL associated with the tolerant ability to submerge stress before utilization as a parent in a breeding program.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

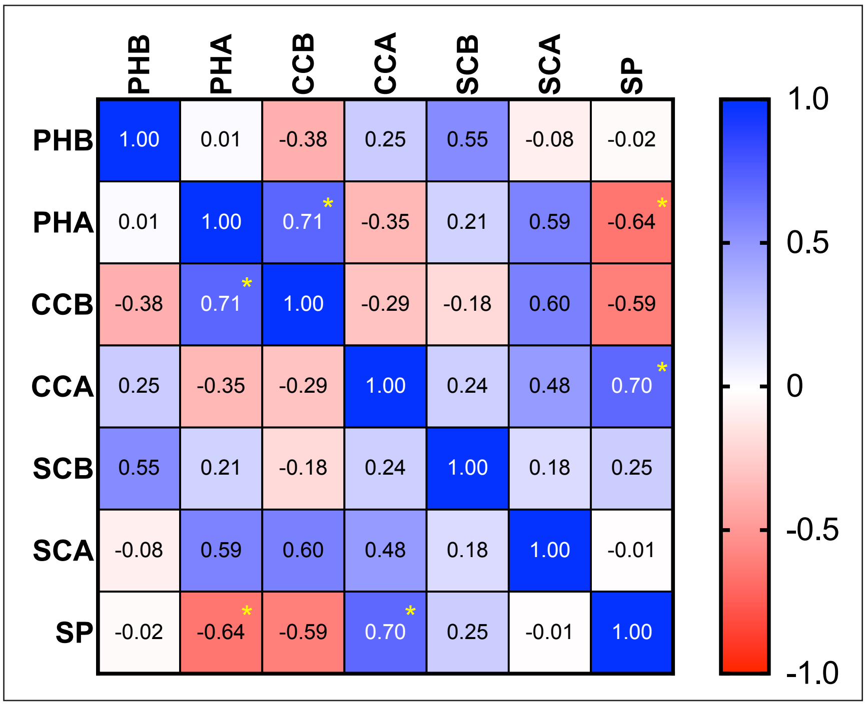

Fig. 2. Correlation matrices between parameters measured in different varieties. PHB = Plant height before submergence; PHA = Plant height after submergence; CCB = Chlorophyll content before submergence; CCA = Chlorophyll content before submergence; SCB = Sugar content before submergence; SCA = Sugar content after; SP = Survival percentage. Asterisks indicate a significant correlation at *p*<0.05.

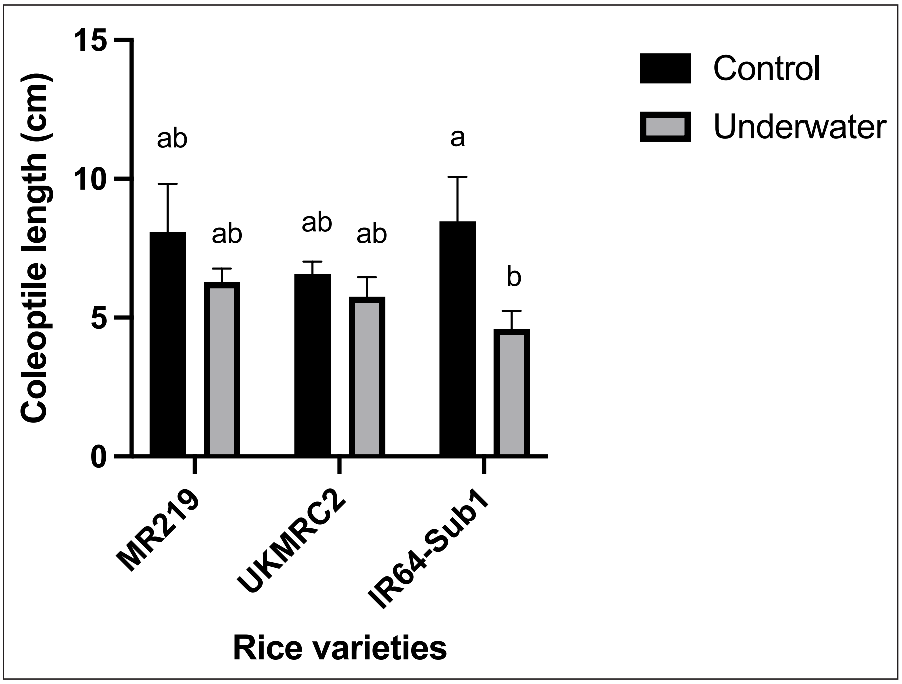

Fig. 3. The coleoptile length of rice varieties geminated in the air (control) and underwater. Each column represents the mean value and SD (*n*=5). Means with different letters are significantly different at *p*<0.05.

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