Rice

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The Relationship between Anaerobic Germination Capacity and Submergence Tolerance in Rice Seedlings

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Abstract

Direct-seeded rice offers multiple advantages, including lower labour costs and a reduced CO_2 footprint. However, the risk of flooding during germination and at the early seedling and vegetative stages is high. Therefore, the capacity for anaerobic germination in waterlogged soils, as well as tolerance to partial and complete submergence, are both essential. It remains unclear whether anaerobic germination and flood tolerance are linked or if they act independently in the environment. Therefore, it is timely to investigate the relationship between these two traits in the context of progressing climate change. We investigated the submergence tolerance of 4-week-old plants of three African landraces, which had previously been shown to possess anaerobic germination capacity. Additionally, we included one submergence-sensitive check and two tolerant checks. These six genotypes were evaluated at three time points: initially (prior to submergence), after three days of submergence, and at the time of desubmergence following 29 days of submergence. We measured survival, key photosynthetic traits (leaf gas films, underwater net photosynthesis, chlorophyll concentration), and carbohydrate reserves. We found that the African landraces tolerant to anaerobic germination all outlived the submergence-sensitive check, 'IR42,' during 29 days of complete submergence. Moreover, all tested genotypes exhibited significant declines over the 29 days of submergence in gas film thickness, underwater net photosynthesis, leaf chlorophyll concentration, and leaf water-soluble carbohydrates and starch. However, no significant differences were observed among the genotypes. The underlying mechanisms of anaerobic germination tolerance in the three African landraces remain unknown, as they do not possess the gene *Anaerobic Germination 1* (*AG1*). Furthermore, it is unclear whether the three genotypes contain the gene *Submergence 1* (*SUB1*); however, *SUB1* confers submergence tolerance only and does not provide tolerance to anaerobic germination. Based on the present study, we cannot rule out the possibility that the novel anaerobic germination tolerance observed in the three African landraces is somehow linked to

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submergence tolerance as well. A thorough bioinformatic analysis is therefore needed to further characterize these landraces.

Keywords Flood tolerance, Hydrophobicity, Leaf gas films, Non-structural carbohydrates, *Oryza sativa*, Photosynthetic parameters, Recovery, Underwater photosynthesis.

Introduction

It may seem surprising that most rice genotypes are unable to germinate even in shallow water, despite rice being a wetland plant. This is due to anoxia in the soil, which develops soon after gas-filled pores become saturated during soil flooding (Ponnamperuma 1972). In rice, anaerobic germination refers to the ability of rice seeds to germinate under waterlogged or oxygen-deficient conditions (Ismail et al. 2009; Miro and Ismail 2013), a critical trait for direct-seeded rice systems (Rauf et al. 2019). Unlike traditional transplanted rice, where seedlings are first grown in nurseries and then transplanted in flooded fields, direct-seeded rice involves sowing seeds directly into the soil (Kumar and Ladha 2011). This method can lead to waterlogging in the early stages, especially in rainfed lowlands or poorly drained fields (Kumar and Ladha 2011). Rice varieties with anaerobic germination ability can withstand these waterlogged conditions, allowing seeds to germinate and establish successfully even in the absence of oxygen (Mondal et al. 2020). This trait is particularly valuable for direct-seeded rice as it reduces the need for precise water management during germination, lowers labour costs, and promotes sustainable water use, making rice production more efficient and resilient in flood-prone areas (Balasubramanian and Hill 2002). Developing rice varieties with strong anaerobic germination potential can, therefore, play a key role in enhancing the viability of direct-seeded rice systems globally.

Quiescence and escape strategies during the vegetative stage of rice are two adaptive responses to flooding that help the plant survive and ensure its reproductive success. Quiescence refers to the ability of certain rice varieties to slow down their metabolism and enter a state of dormancy during submergence (Bailey-Serres and Voesenek 2008). This strategy is underpinned by molecular regulatory pathways that modulate metabolic processes to sustain survival under low oxygen conditions, and it minimizes energy expenditure and oxygen consumption (Xu et al. 2006). By conserving carbohydrates, it allows the plant to withstand relatively short periods under water until the floodwaters recede after which the normal growth is resumed (Ismail et al. 2013). In contrast, the escape strategy involves rapid elongation of the stem or leaves, enabling the rice plant to grow above the surface of the water to access light and oxygen by "snorkelling" (Hattori et al. 2009). This growth acceleration, driven by hormonal signals like ethylene and gibberellins (Kuroha et al. 2018), helps the plant avoid the damaging effects of oxygen deprivation (Mori et al. 2019). While quiescence is often employed by rice varieties in areas with deep, transient floods, escape strategies are common in regions where the flooding is shallower but longer-lasting (Voesenek and Bailey-Serres 2013). Together, these responses enhance resilience of rice to the varying conditions of flooded environments.

Key factors determining survival during submergence include the ability to either conserve or produce carbohydrates and to preserve chlorophyll. Carbohydrate and chlorophyll conservation, observed in submergence-tolerant lines, occurs through the suppression of ethylene synthesis and sensitivity, which slows senescence. Both processes are mediated by *Submergence 1* (*SUB1*) (Xu et al. 2006). Although the quiescence strategy helps conserve chlorophyll and carbohydrates, the initial carbohydrate reserves are not indefinite and will eventually become depleted, leading to a shortage of non-structural carbohydrates during prolonged submergence (Herzog et al. 2018; Ismail et al. 2009). This depletion is further exacerbated by anaerobic metabolism, which produces significantly less ATP per mole of glucose equivalent fermented (Greenway and Setter 1996). The importance of carbohydrate production through underwater photosynthesis is clearly demonstrated in studies comparing plant survival during submergence in light versus darkness, showing that plants exposed to light survive significantly longer (Laan and Blom 1990; Nabben et al. 1999). Consequently, even a small amount of carbohydrate production through underwater photosynthesis can significantly enhance survival during prolonged submergence (Colmer et al. 2014).

During submergence, the superhydrophobic leaves of rice retain a thin gas film under water, facilitating underwater photosynthesis. The superhydrophobicity of the leaves and the resulting gas films represent a key adaptation that allows the plant to continue gas exchange while submerged (Pedersen et al. 2009; Raskin and Kende 1983). This gas film is maintained due to the macro-, micro-, and nano-structural features of the rice leaf surface, which repel water (Koch and Barthlott 2009)–similar to the superhydrophobic properties observed in plants like the lotus (Barthlott and Neinhuis 1997) and *Salvinia* (Barthlott et al. 2010) and in some aquatic insects enabling these to breath under water (Pedersen and Colmer 2012). The macro-structures are formed by the plicate leaves, and most of the gas is trapped in grooves (Lauridsen et al. 2014), and the micro-structures

are made up by the leaf papillae of the rice leaf (Yoo et al. 2011). Finally, the nano-structures consist of dense deposition of wax platelets (Zhang et al. 2016), and the density of these is positively correlated to leaf hydrophobicity and controlled by the *Leaf Gas film 1* (*LGF1*) gene (Kurokawa et al. 2018). The gas film is extremely thin with a median thickness of about 35 µm (Pedersen and Herzog 2024). Nevertheless, the trapped gas layer acts as a barrier, preventing flooding of the substomatal cavities, and provides a large surface area for gas exchange with the floodwater via molecular diffusion (Verboven et al. 2014). Therefore, the gas films are crucial for facilitating underwater photosynthesis and respiration (Colmer and Pedersen 2008). During the daytime, CO_2 dissolved in the floodwater is taken up, while O_2 is exported, preventing photorespiration (Setter et al. 1989). At night, O_2 from the floodwater sustains aerobic metabolism in both the shoots and roots, as it can diffuse longitudinally inside the gas-filled aerenchyma (Winkel et al. 2013). This adaptation allows rice to maintain photosynthetic activity, energy production, and aerobic metabolism during complete submergence, supporting survival and growth in aquatic environments.

Leaf hydrophobicity decreases over time during submergence, leading to the loss of gas films. In a study of four rice genotypes that were fully submerged, the leaf gas films disappeared within 4 to 7 days (Winkel et al. 2014). Once these films were lost, underwater net photosynthesis dropped, and chlorophyll degradation accelerated. However, the duration of gas film retention was not linked to the *SUB1* gene, which was present in two (FR13A and Swarna-sub1) of the tested genotypes (Winkel et al. 2014). A subsequent study found that the thickness of leaf gas films was greater in *SUB1* genotypes compared to those without the *SUB1* locus, a difference attributed to lower expression of the *LGF1* gene in the latter group (Chakraborty et al. 2021). In field conditions, rice benefits from superhydrophobic leaves and the accompanying gas films, aiding underwater photosynthesis and respiration, for approximately one week of complete submergence (Winkel et al. 2014). In contrast, under controlled lab conditions with clean water, these gas films tend to last several days longer (Pedersen et al. 2009). The exact mechanism responsible for the loss of superhydrophobicity underwater remains unclear (Pedersen and Herzog 2024).

Due to the small plant size and typical rainfall patterns in lowland rainfed rice, there is an increased risk of flooding, which can lead to submergence during the early establishment stages. We therefore aimed to test whether there is any relationship between anaerobic germination ability and the tolerance to complete submergence at a later seedling stage in rice. We pursued this aim knowing that, mechanistically, these traits function through opposite mechanisms. Anaerobic germination relies on the continuous breakdown of starch in seeds to maintain higher energy levels for germination under low oxygen conditions, whereas submergence tolerance requires the conservation of carbohydrates through reduced growth and the synthesis of carbohydrates via preserved chlorophyll (Kretzschmar et al. 2015). We used a set of East African rice genotypes (*Oryza sativa*) that had previously been screened for anaerobic germination (Mwakyusa et al. 2023), but never for complete submergence in the vegetative stage. We also included Asian genotypes of *O. sativa* sensitive and tolerant checks as recommended by the International Rice Research Institute (IRRI 2021). We hypothesized that genotypes tolerant to anaerobic germination (essentially the ability to germinate under water) would also have a greater capacity to tolerate complete submergence. We tested the hypothesis by measuring underwater net photosynthesis, leaf gas film retention, chlorophyll degradation, non-structural carbohydrate depletion, and the ability to recover following 29 days of complete submergence, the time point where the submergence-sensitive control plants had all perished.

Table 1 Traits of the six genotypes used in the present submergence study.

Genotype	Anaerobic germination	Submergence	Possessing AG1	Possessing AG2	Possessing SUB1
Kubwa jinga	Tolerant	Tested here	No	??	??
Mapaka wa bibi	Tolerant	Tested here	No	??	??
Wahiwahi	Tolerant	Tested here	No	??	??
IR42	Sensitive	Sensitive	No	No	No
Ciherang-AG1-AG2-Sub1	Tolerant	Tolerant	Yes	Yes	Yes
FR13A	Sensitive	Tolerant	No	No	Yes

Note: 'Kubwa jinga', 'Mpaka wa bibi' and 'Wahiwahi' are Tanzanian landraces screened and found tolerant to anaerobic germination by Mwakyusa et al. (2023). 'IR42' (sensitive) and 'FR13A' (tolerant) are recommended checks for submergence screening (IRRI 2021), and 'Ciherang-AG1-AG2-Sub1' is an improved cultivar with outstanding tolerance to both anaerobic germination and submergence.

Materials and Methods

Plant Material

We included three African landraces ('Kubwa jinga', 'Mpaka wa bibi' and 'Wahiwahi') that were previously screened and found to be tolerant to anaerobic germination (Mwakyusa et al. 2023) along with a sensitive check ('FR13A') and a tolerant check ('Ciherang-Sub1-AG1-AG2') (IRRI 2021). Additionally, 'IR42' was included as a sensitive check for submergence tolerance, while 'FR13A' (already included as a sensitive check for anaerobic germination) also served as a tolerant check for submergence (IRRI 2021), making a total of six genotypes (Table 1). In the present study, previous findings

regarding tolerance and sensitivity to anaerobic germination of the six genotypes were not verified.

One-litre pots (100 × 100 × 100 mm) were filled with compressed, loamy garden soil topped with a 10 mm top layer of fine sand. The pots were waterlogged with tap water (EC 450 µS cm^{-1}) for two days, then drained for two hours before sowing three seeds in each pot. Five replicates of each genotype were used, with each pot serving as the experimental unit. The pots were placed on a waterproof table with a 10 mm layer of tap water to ensure the soil remained moist. After germination, the pots were watered from above twice weekly with a commercial fertilizer (VitaGro 5-1-4, Bayer, Germany) added to tap water following the manufacturer's directions and illuminated (600 µmol photons m^{-2} s^{-1} of photosynthetic active radiation (PAR)) for 18 h per day by high-output LED panels (NS1 BX120, Valoya, Finland) in a light/dark cycle within a glasshouse. Daytime temperatures were maintained at 26–30 °C, with nighttime temperatures above 20 °C.

Survival after Complete Submergence

Tolerance to complete submergence and subsequent recovery were assessed following the protocols of the International Rice Research Institute (IRRI 2021). Five weeks after sowing, the plants were completely submerged in 20 L containers. Since submergence was considered the treatment, the five replicate pots of each of the six genotypes were submerged in separate containers. The submergence solution consisted of 50% tap water and 50% deionized (DI) water, acclimated to 25 °C. To prevent air contact following shoot elongation (snorkelling) in the 50-cm-deep floodwater, the water surface was covered with a transparent plastic foil. The PAR at canopy height was 600 µmol photons m^{-2} s^{-1}, and evaporated water was replenished daily by topping up with DI water. After one week, the pots containing IR42 (the sensitive check for submergence tolerance) were checked daily. Twentynine days after submergence, 5 out of 10 IR42 plants exhibited soft, rotten, smelly tissue at the root-shoot junction, and all genotypes were desubmerged the following day to begin the recovery phase. Growth conditions in air were the same as before submergence. Plants that produced at least one new leaf three weeks after desubmergence were classified as 'survived.'

Leaf Gas Film Thickness

Leaf gas film thickness was measured using the approach described in Raskin and Kende (1983) by determining the volume of gas films by measuring the buoyancy of lamina samples both before and after the removal of gas films. At the three time points (before submergence, after three days of submergence, and at the time of desubmergence) 80 mm long segments were taken from the mid-third of the youngest fully expanded leaf of the main tiller. Initially, buoyancy was measured with the gas films intact using a 4-digit balance with a metal hook underneath (Mettler Toledo ME51). Then, a dilute solution of Triton X (0.1% v/v Triton X-100 in deionized (DI) water), was applied with a soft brush to remove the hydrophobicity of the leaf, eliminating the gas film formation. Buoyancy was measured again after this treatment; a buoyancy of 1 g equals 1,000 mm^3 gas. Segment area was measured using high-resolution scans (Ricoh IM C300) in 600 dpi with subsequent measurements of projected area in ImageJ (Schneider et al. 2012). The average gas film thickness was calculated by dividing the gas film volume (mm^3) by the two-sided area (mm^2), since gas films form on both the adaxial and abaxial sides of rice leaves. In this study, the detection threshold for gas film thickness was approximately 2 µm, so values below this limit were considered as "no gas films present.

Underwater Net Photosynthesis

Underwater net photosynthesis was measured according to Pedersen et al. (2013) at the three time points, i.e., before submergence, after three days of submergence, and at the time of desubmergence. For each leaf replicate, two lamina segments, each about 30 mm in length, were collected from the upper third of the most recently fully expanded leaf blade. Individual segments were placed in glass cuvettes (28 mL) containing the incubation medium (artificial floodwater) and two 4 mm glass beads to aid mixing as the cuvettes rotated vertically on an illuminated wheel in a water bath maintained at 30 °C. Photosynthetically active radiation (PAR) inside the submerged cuvettes was 250 µmol photons m^{-2} sec^{-1}. For the light response curve, the light gradient was obtained by using neutral shading mesh in front of the light source (PureLED Q320W v. 2.0). The exact photon flux was measured by a spherical light sensor (US-SQS/L, Walz, Germany) connected to a light meter (LI-250 A, Li-Cor, the U.S.) The incubation medium for both underwater net photosynthesis and the light response curves contained 0.50 mmol L^{-1} Ca^{2+}, 0.25 mmol L^{-1} Mg^{2+}, 1.00 mmol L^{-1} Cl^-, and 0.25 mmol L^{-1} SO_4^{2-}. To prevent hypoxia, the flasks were incubated in light immediately after the lamina segments were added, and the segments produced O_2 in response to the light. Exact amounts of dissolved CO_2 were achieved by adding specific concentrations of $KHCO_3$ to the incubation medium, with the pH adjusted to 7.20 using HCl to provide a CO_2 concentration of 200 µmol L^{-1} representing typical values of natural floodwaters (Colmer et al. 2011). After incubating for 120–150 min, dissolved O_2 levels were measured in each cuvette using an O_2 optode (Opto-MR, Unisense A/S, Denmark) connected to a pico ampere meter (fx-6 UniAmp, Unisense A/S, Denmark). The sensor

was calibrated at air equilibrium DI water and zero O_2 (ascorbate in an alkaline solution) just before use. Blank cuvettes, incubated under the same conditions but without lamina segments, served as blanks for dissolved O_2. Finally, the projected area of each lamina segment was measured as described under 'Leaf gas film thickness'. Underwater net photosynthesis (μmol O_2 m^{-2} s^{-1}) was calculated as net O_2 evolution (μmol) and divided by incubation time (s) and projected area (m^2).

Photosynthesis in Air

Photosynthetic light response curves were measured in the genotypes 'FR13A' and 'IR42'. Plants were grown with their roots in an aerated nutrient solution and their shoots exposed to air for five weeks. The composition of the nutrient solution (in mM) was as follows: 1.5 $CaSO_4{\cdot}2H_2O$, 7.5 MES (buffer), 0.4 $MgSO_4{\cdot}7H_2O$, 3.75 KNO_3, 0.625 NH_4NO_3, 0.1 $Na_2O_3Si{\cdot}5H_2O$, 0.05 Fe-EDTA, and 1.0 micronutrients. The micronutrients composition (in mM) was: 0.05 KCl, 0.025 H_3BO_3, 0.002 $MnSO_4{\cdot}H_2O$, 0.002 $ZnSO_4{\cdot}7H_2O$, 0.0005 $CuSO_4{\cdot}5H_2O$, 0.0005 $Na_2MoO_4{\cdot}2H_2O$, and 0.001 $NiSO_4{\cdot}7H_2O$. The pH was adjusted to 6.5 using 2 M KOH. Measurements were taken from three-week-old plants using an infrared gas analyzer system (LI-6800, LI-COR Biosciences Inc., Lincoln, NE, USA). Data were collected from the upper third of the most recently fully expanded leaf at seven irradiance levels (0, 25, 50, 100, 250, 500, and 1500 μmol photons m^{-2} s^{-1} in the PAR spectrum), with CO_2 concentration of 425 ppm and relative humidity of 70–80%. All measurements were conducted between 10:00 and 15:00 h.

SPAD Analyses

To assess overall shoot tissue integrity, SPAD measurements were conducted on the middle section of the youngest fully expanded leaf at three time points: prior to submergence, following desubmergence, and at the end of the recovery phase. The SPAD measurements were taken using a SPAD meter (SPAD-502 Plus, Konica Minolta, Japan).

Analysis of Non-structural Carbohydrates

For each time point, i.e., before submergence, after three days of submergence, and at the time of desubmergence dry leaf samples (youngest fully expanded leaf) were ground with a ball mill (MM500, Retsch, Haan, Germany), directly into microtubes, to obtain a fine powder (Ø < 150 μm). Total soluble non-structural carbohydrates and starch extraction followed the method proposed by Quentin et al. (2015) and with minor modifications recommended by Casolo et al. (2023).

Total water-soluble non-structural carbohydrates were extracted by adding 250 μL of 50 mM Tris-HCl (pH 7.5). After the resuspension, the samples were incubated at 80 °C for 30 min, centrifuged (11,000 *g* for 2 min), and the supernatant transferred to a new microtube. The suspension contained all water-soluble carbohydrates: sugars (mainly glucose, fructose, sucrose and maltose) and maltodextrins. The pellet containing starch was resuspended with 250 μL of 10 mM of Tris-HCl (pH 6.9) + 250 μL of 25 mM sodium acetate (pH 4.6) and the gelatinization was performed by boiling samples in the oven at 100 °C for 1 h. Then, 100 U/samples of α-amylase and 25 U/samples of amyloglucosidase were added and samples were incubated for 48 h at 70 °C to allow starch hydrolysis. The concentration of the total soluble non-structural carbohydrates was estimated with the anthrone assay (Yemm and Willis 1954) as hexose equivalents (g g-1 DM) by comparing sample absorbance with known glucose amounts. The samples were measured at 620 nm using a spectrophotometer (VICTOR, Perkin Elmer).

Starch measurement followed the enzymatic method of Landhäusser et al. (2018) with adaptations for low concentrations described in (Gargiulo et al. 2024). Briefly, glucose from starch hydrolysis was converted into NADH (stochiometric rate 1:1) by adding 0.2 U/sample of hexokinase and 0.5 U/sample of glucose 6-P dehydrogenase. The NADH produced was measured with a spectrophotometer at 340 nm and compared with amylose standards.

Data Analysis

GraphPad Prism software (v. 10.3.1) was used for statistical analyses. Two-way ANOVA followed by Tukey's test was used to test the effect of genotype and submergence tolerance. All data met the assumption of normality (Shapiro-Wilk test) and homoscedasticity (Bartlett's test) without requiring data transformation. The figure legends provide details on the tests used and the levels of significance.

Results

The main hypothesis of the study was to test whether genotypes tolerant to anaerobic germination also possess a capacity to tolerate complete submergence. To evaluate this, we completely submerged sensitive controls ('IR42'), tolerant controls ('FR13A' and 'Ciherang-Sub1-AG1-AG2'), and three African genotypes ('Kubwa jinga', 'Mpaka wa bibi' and 'Wahiwahi') previously screened and identified as tolerant to anaerobic germination (Mwakyusa et al. 2023). After 29 days of complete submergence, the sensitive control ('IR42') exhibited clear signs of tissue disintegration at the root-shoot junction. Following desubmergence, the plants were left to recover for three weeks. None of the sensitive controls recovered, whereas all tolerant controls produced at least one new leaf, achieving 100% survival (Fig. 1A, B). Interestingly, all

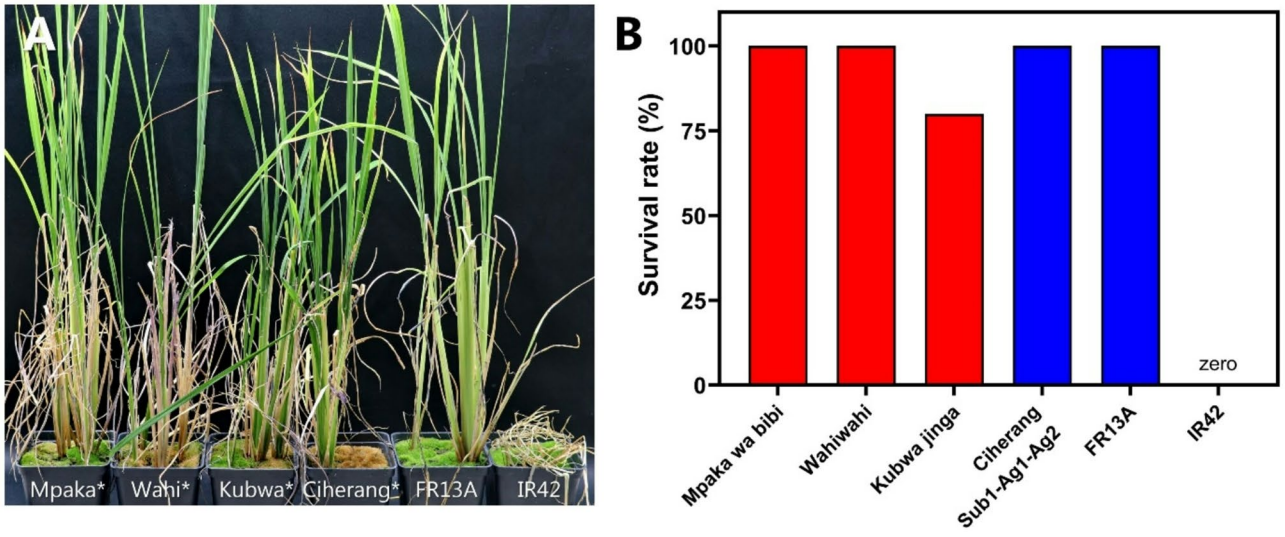

Fig. 1 Survival of 5-week-old rice genotypes after 29 days of complete submergence. During complete submergence, the plants were inundated in a 1:1 mixture of tap water and deionized water, with elongation into the air prevented by a thin, transparent plastic foil covering the surface. (A) a photo of six genotypes tested after three weeks of recovery under drained conditions with one of five typical replicates shown. (B) survival rates of the three African genotypes (red bars), the two submergence-tolerant controls (blue bars), and the submergence-sensitive control (bar not visible, as all individuals failed to recover). * name of genotype truncated; see (B) for full names.

three African genotypes tolerant to anaerobic germination also survived complete submergence, although one genotype had a slightly lower survival rate (80%, Fig. 1B).

In an attempt to gain a better mechanistic understanding of the underlying reasons for the observed differences in survival, we conducted several diagnostic tests of the shoot tissue viability before submergence (initial conditions), after three days of submergence, and at the time of desubmergence.

Leaf gas films of rice are known to significantly enhance underwater gas exchange by facilitating increased CO_2 uptake and O_2 production in light, as well as greater O_2 consumption in darkness (Winkel et al. 2013). Therefore, we measured leaf gas film thickness in all cultivars prior to submergence, after three days of submergence, and at the time of desubmergence. Initially, the gas film thickness was 16.7 ± 0.6 (mean ± SE) µm, and did not change significantly after three days of submergence (17.8 ± 1.0). However, after 29 days of submergence, the leaves of all six genotypes became hydrophilic, and leaf gas films had therefore completely degraded (Fig. 2A).

Due to the positive effect of leaf gas films on underwater gas exchange (Verboven et al. 2014) and chlorophyll's critical role in light harvesting, we both measured underwater net photosynthesis and soil-plant analysis development (SPAD, relative chlorophyll content). These two parameters showed identical trends, i.e., decline with time of submergence. The decline was strongly significant, but there was no effect of genotype, and only a minor, but significant, effect of submergence time × genotype (Table 2; Fig. 2B, C). The interpretation of the interaction is that the effect of submergence time on underwater net photosynthesis and SPAD was not the same for all genotypes. The decline in underwater net photosynthesis over time during submergence is not only due to the loss of leaf gas films, which impairs gas exchange, but also to chlorophyll degradation. This is supported by the strongly significant positive correlation between underwater net photosynthesis and SPAD values (Fig. 2D).

The submergence environment is characterized by low light (Vervuren et al. 1999) and restricted gas exchange (Colmer et al. 2011), even in the presence of leaf gas films. We therefore constructed light response curves for underwater photosynthesis in FR13A (the submergence-tolerant check) and IR42 (the submergence-sensitive check), and for comparison, we also measured light response in air (Fig. 3). In air, the light response of FR13A and IR42 was similar in terms of light use efficiency (ϕ) and high maximum photosynthesis (P_{max}) with neither of these differing among the two genotypes. In comparison, under water ϕ and P_{max} were only 18 and 9%, respectively, of those in air in both genotypes assuming a photosynthetic quotient of 1.0 (Ulqodry et al. 2016) (Table 3). Likewise, the dark respiration differed significantly in air and water (Table 3) indicating that the O_2 flux into the leaf to support dark respiration under water was limited by slow O_2 diffusion.

Non-structural carbohydrates in paddy rice are known to decline substantially in response to submergence (Ismail et al. 2009). Therefore, we measured both water-soluble non-structural carbohydrates (i.e., sugars and

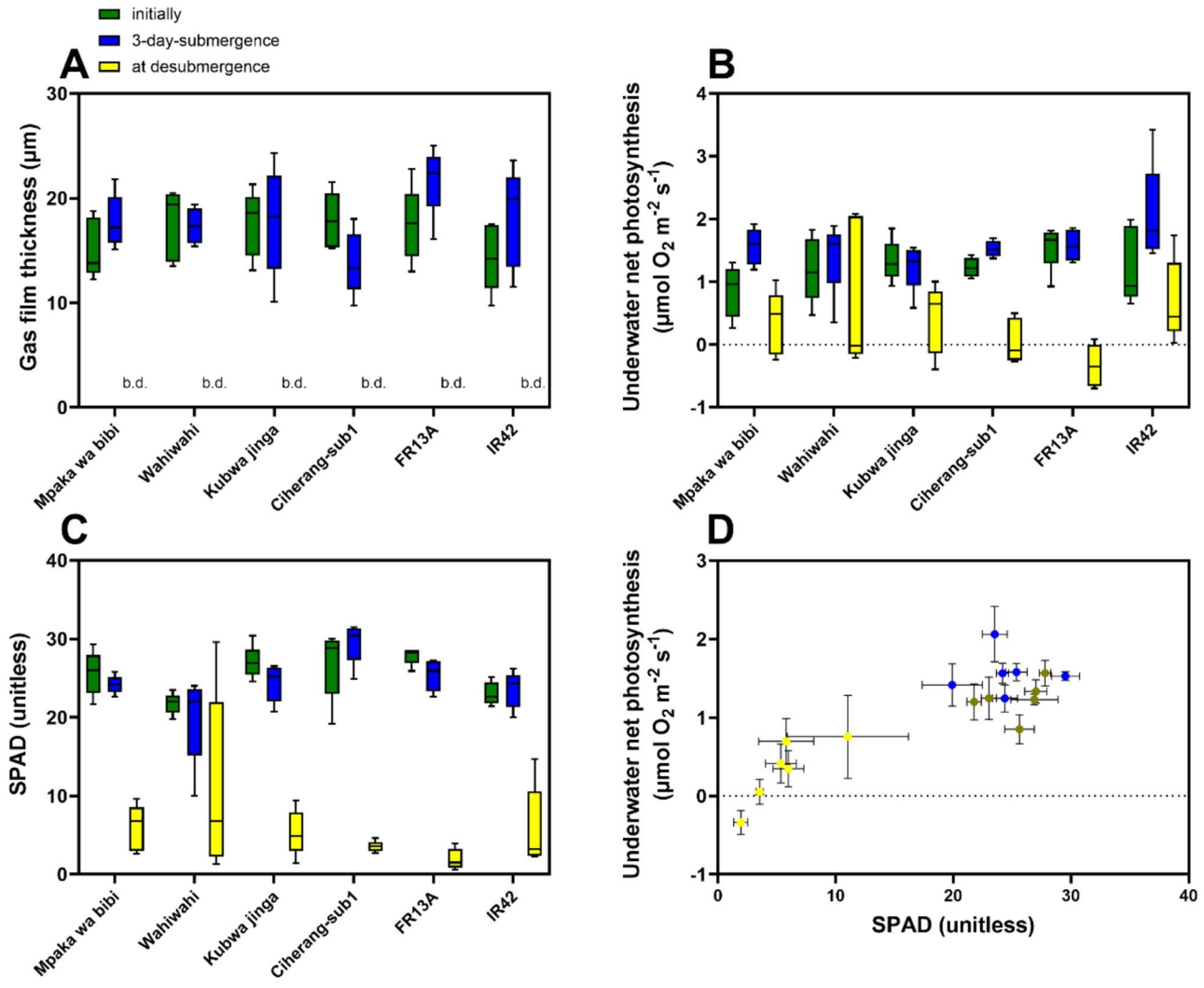

Fig. 2 Shoot traits of six rice genotypes prior to submergence (green), after 3 days of submergence (blue) and at the time of desubmergence (yellow). (A) leaf gas film thickness, (B) underwater net photosynthesis, (C) SPAD values, and (D) the correlation between underwater net photosynthesis and SPAD. The seedlings were 5-week-old at the time of submergence, and they were submerged for 29 days. At each time point, measurements were taken using the youngest fully expanded leaf. The box-and-whisker plots display the median (horizontal line), the first and third quartiles (box), and the minimum and maximum values (whiskers) based on five true replicates, with each seedling serving as the experimental unit. In the correlation panel, each point represents the mean ± SE of the five replicates. Spearman correlation coefficient = 0.73, $P < 0.0003$. SPAD = soil-plant analysis development (relative chlorophyll content), b.d. = below detection limit. See Table 1 for ANOVA results; for clarity, *post hoc* analyses are not shown, but these are available in Table S1.

maltodextrins) and starch at three time points (before submergence, after three days of submergence, and at the time of desubmergence). As hypothesized, both non-structural carbohydrates and starch declined significantly over the 29 days of submergence (Fig. 4A, B; Table 2). For non-structural carbohydrates, there was also a significant effect of genotype and a submergence × genotype interaction, indicating that the genotypes did not respond to submergence in a uniform manner. However, due to missing data for time point No. 2 (3 days after submergence), two of the genotypes had to be excluded from the ANOVA analysis, so these results should be treated with caution. For starch, we obtained a complete dataset and observed a significant effect of genotype, but no interaction between submergence and genotype (Table 1). For both non-structural carbohydrates and starch, the concentration was critically low at the time of desubmergence. The effect of genotype revealed a significant increase of starch concentration during 3-days submergence only in genotypes sensitive to anaerobic germination, i.e., 'FR13A' and 'IR42' (Table S1).

The substantial decline in underwater net photosynthesis over the submergence period (Fig. 2B) prompted us to investigate its correlation with non-structural carbohydrates and starch. As anticipated, we found a significant positive correlation between underwater net photosynthesis and both non-structural carbohydrates and starch (Fig. 4C, D), suggesting that the depletion in carbon

Table 2 Leaf trait versus source of variation, percentage of total variation explained by each variable, and the P value for two-way ANOVA

Variable	Source of variation	% of total variation[1]	*P* value
Leaf gas film thickness	Submergence time	88.5	< 0.0001
	Genotype	0.9	n.s.
	Submergence time × genotype	2.3	0.0485
Underwater net photosynthesis	Submergence time	47.7	< 0.0001
	Genotype	3.8	n.s.
	Submergence time × genotype	10.4	0.0492
SPAD	Submergence time	81.3	< 0.0001
	Genotype	0.7	n.s.
	Submergence time × genotype	6.1	0.0005
Non-structural carbohydrates[2]	Submergence time	53.2	< 0.0001
	Genotype	5.1	0.0251
	Submergence time × genotype	17.4	< 0.0001
Starch	Submergence time	30.4	< 0.0001
	Genotype	0.5	n.s.
	Submergence time × genotype	8.3	n.s.

Note: [1]The total variation does not sum to 100% due to unexplained variation. [2]Due to missing values 3 days after submergence, the ANOVA was conducted excluding the two genotypes, 'Mpaka wa bibi' and 'IR42'. SPAD = soil-plant analysis development (relative chlorophyll content), n.s. = non-significant

reserves is strongly linked to the decline in underwater net photosynthesis.

Discussion

In the present study, we tested the hypothesis that genotypes that had previously been shown to tolerate anaerobic germination (Mwakyusa et al. 2023) would also have a greater capacity to tolerate complete submergence. Using three of the genotypes identified as tolerant to anaerobic germination, we found that all of them survived 29 days of complete submergence, similar to the tolerant checks included in our study. However, none of the diagnostic tests used– underwater net photosynthesis, leaf gas films, chlorophyll content, or leaf carbon reserves– were associated with the ability to survive underwater for nearly a month. Below, we discuss these findings in the context of current knowledge and provide avenues for further investigation to better understand the drivers behind tolerance to complete submergence.

In Asian rice, anaerobic germination and submergence tolerance are governed by different QTLs. For anaerobic germination, two major QTLs and their associated genes have been identified: *AG1* on chromosome 9 and *AG2* on chromosome 7 (Angaji et al. 2010; Tnani et al. 2021). Long-term submergence tolerance, on the other hand, is primarily controlled by the *SUB1* QTL (Mudhale et al. 2024; Xu et al. 2006), which includes at least three ethylene response factors and is also located on chromosome 9 (Bailey-Serres et al. 2010). In a study where 11 African landraces out of 208 genotypes were identified as tolerant to anaerobic germination, none of these contained *AG1* suggesting these landraces contain genetically novel tolerances (Mwakyusa et al. 2023). Unfortunately, the presences of neither *AG2* nor *SUB1* was tested for in that study. Thus, it is presently unknown if the three landraces included in the current study contains *AG2* or *SUB1*, but *AG1* is not present in the three.

All four genotypes tolerant to anaerobic germination as well as 'FR13A' survived 29 days of complete submergence, and only the submergence sensitive check, 'IR42', perished. This is a rather long submergence event, and the capacity to survive it may seem surprising at first glance. However, the survival conditions were favourable as the plants were submerged in clear water with excellent light conditions (initially at 600 µmol photons m^{-2} s^{-1}, which is equivalent to 25% of tropical sunlight at noon), which would have supported rates of underwater net photosynthesis near the maximum capacity (Figs. 2B and 3B), at least initially when the leaf gas films were still intact. As the submergence progressed, the water became increasingly more turbid due to blooms of planktonic algae, and the dying leaves were also colonized by filamentous algae both of which would have competed for light and CO_2. In a field situation, the floodwater is typical much more turbid already at the onset of submergence, and this is likely the main reason why 'IR42' or other flood sensitive checks dies much earlier in most studies on submergence tolerance of rice (Chakraborty et al. 2021; Ismail et al. 2013; Niroula et al. 2012).

A clear link between survival during complete submergence and the capacity to conserve carbohydrates has already been established. Conservation can be achieved through a quiescence response to submergence, where leaf and/or stem elongation are greatly restricted. In rice, this quiescence response is controlled by the *SUB1* locus (Xu et al. 2006) as noted above. Genotypes containing *SUB1* generally maintain higher carbohydrate reserves towards the end of a submergence event compared with genotypes lacking this trait (Bhaduri et al. 2020; Das et al. 2005; Winkel et al. 2014). In addition to conserving non-structural carbohydrates through the suppression of elongation, carbohydrates may also be produced during submergence via underwater net photosynthesis. Enhanced gas exchange facilitated by leaf gas films significantly enhance sugar accumulation during complete submergence (Pedersen et al. 2009). Moreover, studies have shown that genotypes with *SUB1* tend to have thicker leaf gas films that persist longer (Chakraborty et al. 2021). Therefore, the beneficial function of *SUB1* may involve dual actions: conserving carbohydrates by

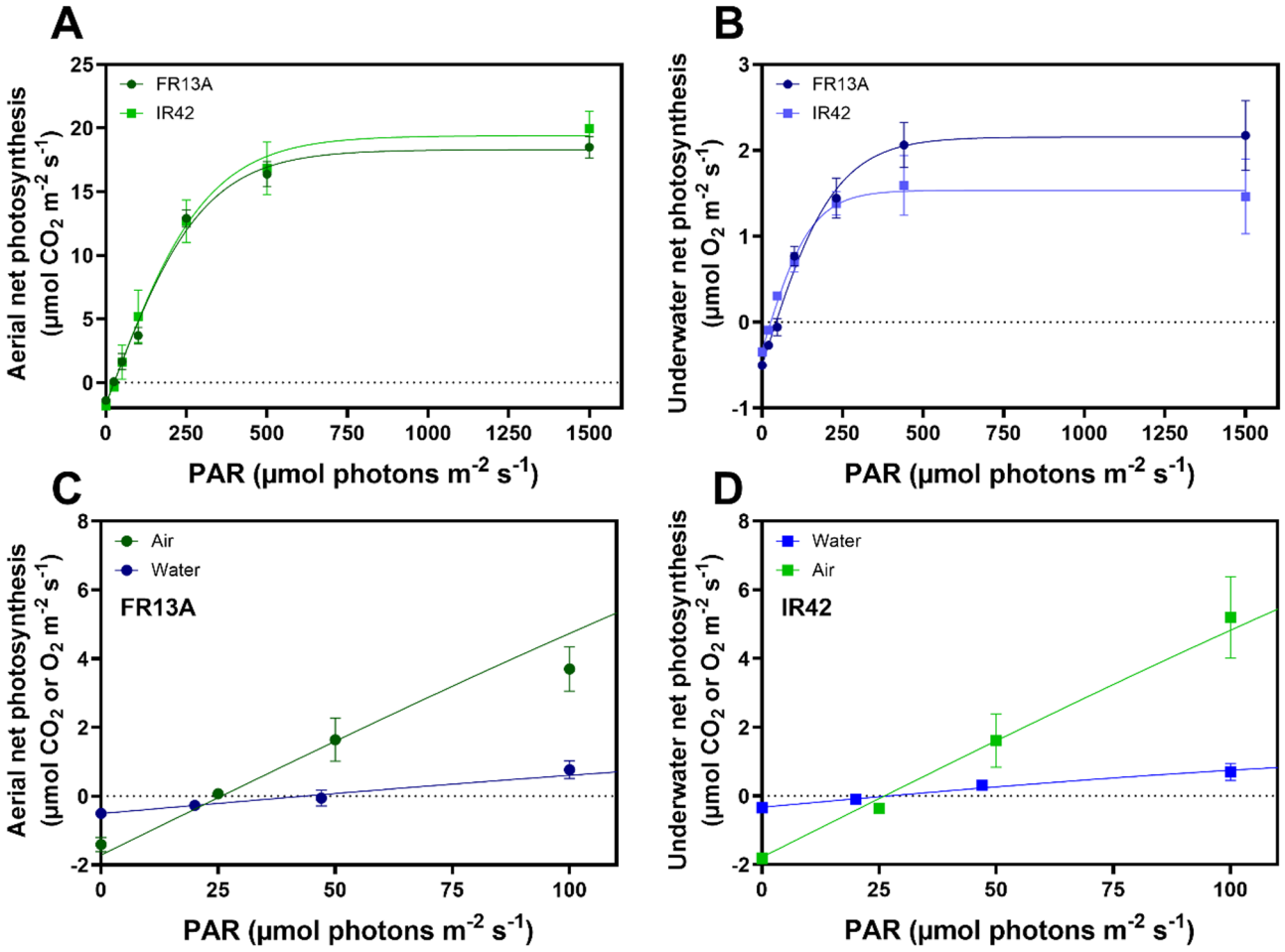

Fig. 3 Light versus photosynthesis in two 5-week-old rice genotypes: 'FR13A' (submergence-tolerant) and 'IR42' (submergence-sensitive). (A) photosynthesis in air with 425 ppm CO_2 and 70–80% relative humidity, (B) photosynthesis in water with 225 µmol CO_2 L^{-1}, (C) close-up of rates of FR13A (C) and IR42 (D) to facilitate direct comparison of R_D and initial slope in air and water. Rates in both air and under water were taken at 30 °C. R_D, P_{max} and ϕ were both estimated using the photosynthesis versus light function by Jassby and Platt (1976); see Table 3 for these key photosynthetic parameters.

Table 3 Key photosynthetic parameters in air or under water for two rice genotypes 'FR13A' (submergence-tolerant) and 'IR42' (submergence-sensitive).

	'FR13A'		**'IR42'**	
	Air	Water	Air	Water
R_D	-1.72[a]	-0.51[b]	-1.78[a]	-0.33[b]
Φ	0.067[a]	0.012[b]	0.068[a]	0.012[b]
P_{max}	20.0[a]	2.66[b]	21.2[a]	1.86[b]

Note: units of R_D and P_{max} are µmol CO_2 m^{-2} s^{-1} and µmol O_2 m^{-2} s^{-1} in air and water, respectively. For Φ, the units are µmol CO_2 $µmol^{-1}$ photons and µmol O_2 $µmol^{-1}$ photons in air and water, respectively. Different superscript letters indicate statistical significant differences ($P < 0.05$, based on the Jassby and Platt (1976) model.

restricting elongation and increasing carbohydrate production through the retention of leaf gas films, both of which contribute to a superior carbohydrate status towards the end of a submergence event. However, in the present study, all genotypes had depleted the carbohydrate reserves to a critical point after 29 days of complete submergence with no significant difference between the six genotypes in non-structural carbohydrates, and only a minor effect ($P = 0.016$) in starch content but with no differences between genotypes (Fig. 4A, B, Table S1). While the focus in the study was on the youngest fully expanded leaf lamina, the stem (or rather the leaf sheaths) also stores carbohydrates in the aboveground tissues of rice (Kato et al. 2019). It is therefore possible that we missed important signals in carbohydrates as a consequence of our focus on the leaf lamina and its more direct association with photosynthetic production of carbohydrates. To our knowledge, no previous studies have specifically examined carbohydrate concentrations in the meristematic tissues of shoots and roots following depletion during complete submergence. We therefore propose that future research on submergence tolerance should focus on these tissues, as their vitality is likely crucial for survival, similar to how the shoot apical meristem of seagrasses has been shown to be a key determinant of

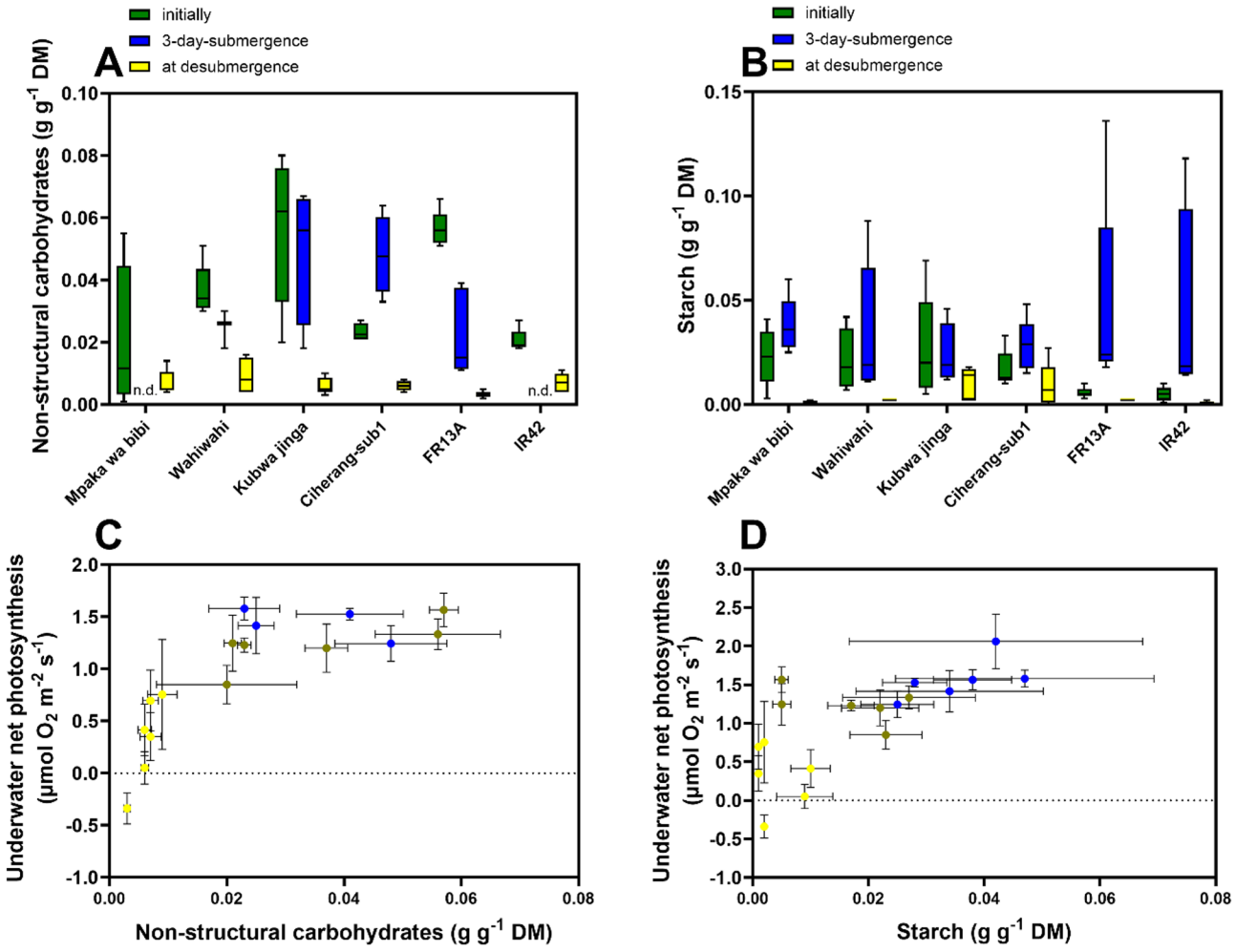

Fig. 4 Non-structural carbohydrates (A), starch (B), and the relationship with underwater net photosynthesis, (C-D), in six rice genotypes prior to submergence, after 3 days of submergence and at the time of desubmergence. Colour coding in (C-D) reflects the three time points shown in the legends of (A-B). In (C), Spearman correlation coefficient = 0.85, $P < 0.0001$, in (D), Spearman correlation coefficient = 0.75, $P < 0.0003$. See Table 1 for ANOVA results; for clarity, *post hoc* analyses are not shown, but these are available in Table S1. In (A), n.d. = 'not determined' due low amounts of plant material.

survival under low oxygen conditions in aquatic environments (Borum et al. 2005; Greve et al. 2003).

Maintenance of photosynthesis during the early stages of submergence (Fig. 2D) ensures a high concentration of non-structural carbohydrates in rice plants (Fig. 4), which are subsequently used to sustain metabolism (Panda and Sarkar 2014) and growth (Nurrahma et al. 2021; Wang et al. 2016). The increase in starch observed after three days of submergence in the sensitive check genotype, but not in submergence-tolerant genotypes, suggests that starch accumulation in leaves may result from limited translocation of sugars from source to sink organs. Such an impairment can have detrimental effects on growth and survival. On the other hand, soluble carbohydrates can help plants tolerate submergence by protecting cell membranes (Herzog et al. 2016). In many species within the Poaceae family, this crucial role is played by short water-soluble polymers such as fructans (Valluru and Van den Ende 2008). However, rice does not biosynthesize fructans (Fretzdorff and Welge 2003; Verspreet et al. 2015), and the high levels of water-soluble carbohydrates observed in our study suggest an alternative strategy, such as the production of maltodextrins. Maltodextrins are starch degradation products that have recently been suggested to play a role in drought tolerance in grapevine (Vuerich et al. 2023). Although rice is a source of maltodextrins in the food industry (Amin et al. 2015; Wang and Wang 2000), their physiological role in the plant remains unexplored. Rice maltodextrins, characterized by short glucose chains (Wang and Wang 2000) represent an important source of sugars that can be easily transported throughout the plant. This aspect should be considered in future research investigating the role of non-structural carbohydrates in rice stress tolerance.

The carbohydrate production during complete submergence is the result of CO_2 fixation by the leaves. We

therefore monitored leaf gas film thickness during the first three days of submergence. However, the six genotypes showed no differences in gas film retention after three days of submergence (Fig. 2A). Consequently, it was not surprising that underwater net photosynthesis followed the same trend, with no differences in photosynthetic rates between the initial measurements and those taken after three days of submergence (Fig. 2B). However, underwater net photosynthesis was still significantly suppressed compared to rates in air, with Pmax reaching only approximately 10% of the rates observed in air. This reduction was consistent regardless of whether it was evaluated in the submergence-sensitive check ('IR42') or the tolerant check ('FR13A') (Fig. 3A, B). The low photosynthetic rates under water can be attributed to the 10,000-fold slower gas diffusion in the liquid phase compared to air, resulting in substantial CO_2 limitation of photosynthesis. While this CO_2 limitation can be partly compensated for by increasing CO_2 availability, we had already elevated the CO_2 concentration to 15 times above air-equilibrium levels (Materials and Methods) to simulate typical floodwaters (Colmer et al. 2011). Nevertheless, the leaf lamina appeared flaccid, and senescence was progressing by the time of desubmergence. This was clearly reflected in several negative rates of underwater net photosynthesis (indicating net O_2 consumption) and low chlorophyll concentration, and both traits showed high variation at the time of desubmergence (Fig. 2B, C).

The vast majority of our current knowledge on abiotic stress tolerance comes from studies on Asian rice, *O. sativa*, whereas little is known about potential novel tolerance traits in rice of African origin, including its native landraces, wild relatives and the African rice sub-species, *O. glaberrima*. The latter was domesticated largely in west Africa about 3000 years ago, and is known for its tolerance of several abiotic stresses and resistance to common diseases in Africa (Linares 2002). These genetic resources are yet to be exploited to enhance adaptation of modern rice varieties to biotic and abiotic stresses, currently being aggravated by climate change adversities, including flooding.

A critical next step following this study is a comprehensive analysis of the newly identified landraces that likely lack the *Sub1* locus. Observations of landraces exhibiting atypical tolerance to flooding and complete submergence date back to the early 1950s, with more systematic screening efforts beginning in the 1970s. Among the accessions recognized for their notable resilience to complete submergence were FR13A and FR43B from Orissa, India, as well as Kurkaruppan, Goda Heenati, and Thavalu from Sri Lanka (Bailey-Serres et al. 2010). Therefore, the likelihood that these African rice landraces possess novel forms of submergence tolerance is high. Additionally, it is essential to assess the quantitative trait loci and genes associated with both aerobic germination and submergence tolerance in these landraces, particularly under the assumption that they do not carry *Sub1*. These landraces represent valuable donors of novel traits and genes with significant potential for both scientific advancements and genetic improvement. Harnessing their genetic diversity could provide new opportunities to enhance rice resilience in regions currently underutilized or at risk due to abiotic stresses and climate change. This work will be key in improving and sustaining food security, particularly in sub-Saharan Africa.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12284-025-00806-3.

Supplementary material 1.

Acknowledgements
We greatly appreciate the screening work for anaerobic germination conducted by Lupakisyo Mwakyusa at Sokoine University of Agriculture. This work was supported by Danida (grant No. 19-03-KU and 25-07-KU). J.d.l.C.J. was funded by the European Union under a Marie Skłodowska-Curie Postdoctoral Fellowship (grant No. 101061962, SmartRoots) and by the Novo Nordisk Foundation (grant No. NNF23OC0084801).

Author Contributions
DLED, ZS & OP conceived the study. DLED, AGA, ZS, SG, VC & OP conducted the experiment. OP analysed the data and drafted the figures. AMI, SN-M, SG, VC, ZS, JdlCJ & OP drafted the text. All authors approved the final submission.

Funding
Open access funding provided by Copenhagen University

Data Availability
Data is provided within the manuscript or supplementary information files.

Declarations

Competing Interests
The authors declare no competing interests.

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Received: 18 February 2025 / Accepted: 23 May 2025
Published online: 28 May 2025

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