UNIVERSITY OF COPENHAGEN
FACULTY OF SCIENCE

Leaf gas film thickness and persistence during complete submergence in two wild rice species: *Oryza australiensis* and *Oryza barthii*

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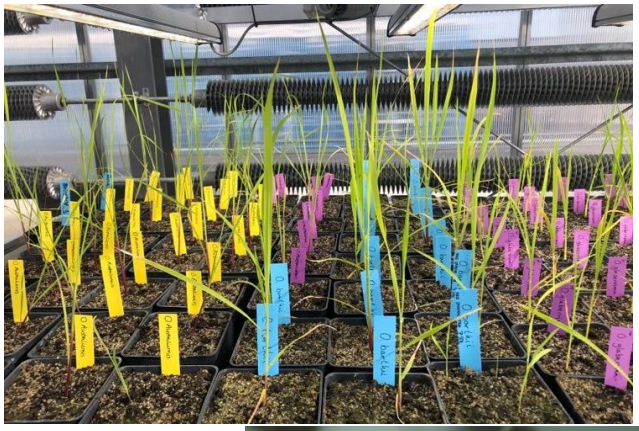

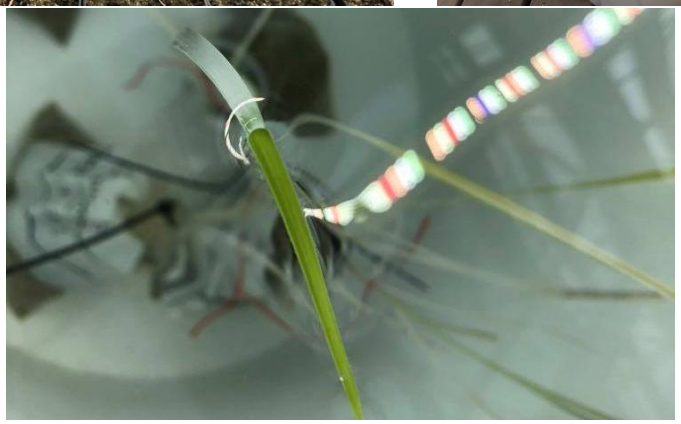

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Abstract

Climate resilient rice varieties are of great importance as floods are predicted to occur more frequently in future climate projections. During floods, terrestrial plants experience a 10^4-fold slower gas exchange and a reduced light availability which limits photosynthesis and respiration. Further, waterlogging of soils can cause hypoxia or anoxia in plant roots. Terrestrial wetland plants have evolved traits related to flood tolerance including formation of aerenchyma, root barrier to radial oxygen loss, shoot elongation, production of new 'semi-aquatic' leaves, and formation of leaf gas film (LGF). 22 wild rice species are claimed as an important genetic resource to identify flood tolerant traits. However, studies of wild rice species are scarce.

This study investigates the LGF thickness, leaf porosity and underwater net photosynthesis (P_N) of two wild rice species: *O. australiensis* and *O. barthii* during complete submergence. Leaf segments for measurements were harvested at day 0, 2, 4, 8 and 17. There was no significant difference in LGF thickness and persistence between *O. australiensis* and *O. barthii* even though they originate from different habitats. They both formed thinner LGF than previous studied rice cultivars (*O. sativa*) but retained their LGF for a longer period than other rice cultivars, which may be due to the conditions of the floodwater used in this study. LGF thickness, porosity and underwater P_N were all negatively affected by submergence time which had possible negative impacts on plant yield and/or survival. This study suggests that the formation of a thick LGF as a flood tolerant adaptation may not have been advantageous for *O. australiensis* and *O. barthii*. A screening of 20 other wild rice species and their LGF thickness and persistence may be helpful in improving flood tolerance in rice.

Introduction

Climate change causes a need for flood tolerant rice varieties

Rice yield and food security are threatened by climate change. Recent climate projections predict changes in precipitation patterns, rising temperature and more extreme weather events leading to climatic disasters e.g., drought, high temperatures, floods, and salinity stress (Lesk et al., 2016, Oladosu et al., 2020, Shi et al., 2021). Rice, which is feeding more than half of the world's population, is one of the most flood threatened crops due to major cultivating areas in rainfed lowland and flood-prone regions (Bailey-Serres et al., 2012, Oladosu et al., 2020). Nearly 20-22 million ha of rainfed lowland areas in Asia and Africa are anticipated to be affected by flash floods (Das et al., 2009, Mackill et al., 2012). Consequently, this underlines the importance of developing climate resilient rice varieties, that are adapted to abiotic stresses e.g., floods. Floods, which causes partially or fully submergence, can broadly be distinguished by two types: flash flood and stagnant flooding (Kumar et al., 2021). This study focused on flash floods which are a rapid complete inundation of the plants lasting from several days up to two weeks.

Submergence tolerance in rice

Rice is considered a flood tolerant crop. However, only few rice cultivars show tolerance to prolonged submergence, with most of them dying following 14 days of complete submergence (Menguer et al., 2017). Out of 24 wild rice species, two rice species have been cultivated: *Oryza sativa,* the Asian cultivar and *Oryza glaberrima*, the African cultivar (Atwell et al., 2014). However, the genetic diversity of *O. sativa* is found to be less than half the diversity in wild relatives of *Oryza*, and *Oryza glaberrima* has even less genetic diversity than *Oryza sativa* (Atwell et al., 2014). Therefore, wild rice species are claimed as an important genetic resource for improvement of future rice cultivation as wild rice species are distributed pan-tropical across several biomes (Atwell et al., 2014, Menguer et al., 2017).

Challenges during submergence for terrestrial plants

Terrestrial plants suffer substantial damage in plant function or even premature death when partially or completely submerged. This is mainly due to a 10^4-fold slower diffusion of dissolved gases in water as compared with in air, in which direct gas exchange between submerged tissues and environment is strongly hampered (Armstrong, 1979, Colmer, 2003). As a result, photosynthesis and

respiration are limited, as CO_2-uptake to chloroplasts are required for photosynthesis during day, and entry of O_2 is required for respiration (Colmer and Voesenek, 2009). Photosynthesis is also restricted by light as surface reflection, back-scattering and absorption by water and suspended particles influence light intensity (Mommer and Visser, 2005, Colmer and Voesenek, 2009). Waterlogged plant roots may experience hypoxia or even anoxia with strong consequences for roots and plant survival (Drew, 1997). As a result of reduced soils (low redox potential due to absence of O_2), roots may experience higher CO_2 concentrations in the root zone, accumulation of phytotoxins (Fe^{2+}, Mn^{2+} and H_2S) and metabolites (e.g., acetic acid and butyric acid). Plants, that have adapted to transient or long-term floods, have evolved traits for flood tolerance as they are of great importance for plant survival.

Submergence tolerance in wetland plants

Several metabolic and morphological mechanisms to cope with the adverse effects of submergence have evolved as plants are sessile organisms, and terrestrial wetland plants typically grow in low elevated areas with waterlogged soils and/or areas frequently flooded (Mommer and Visser, 2005, Bailey-Serres and Voesenek, 2008). The response to complete submergence can be classified into: The Low Oxygen Quiescence Syndrome (LOQS) and the Low Oxygen Escape Syndrome (LOES) (Colmer and Voesenek, 2009). In LOQS, elongation of shoots are depressed, conserving carbohydrates for metabolic processes, which prolong survival during submergence (Colmer and Voesenek, 2009). *SUB1*, a gene, is an example of the LOQS response which is now used in several rice cultivars to improve flood tolerance (Bailey-Serres et al., 2010). However, Mackill et al. (2012) suggests, that new varieties that combine the *SUB1A* gene with other flood tolerant traits, are needed to improve submergence tolerance in flood-prone areas. In LOES, shoots, stems and leaves are stimulated to elongate, escape submergence, and maintain shoots above water level allowing photosynthesis and respiration in air (Bailey-Serres and Voesenek, 2008, Hattori et al., 2011). This strategy requires high amounts of energy and less energy for metabolic processes. Consequently, this may not be efficient use of energy during flash floods as shoots tend to lodge when floods recedes (Mackill et al., 2012).

Many wetland species can form aerenchyma, air-filled tissues in shoots and roots when exposed to waterlogged soils or submergence. Aerenchyma enhances the internal longitudinal movement of gases within the plant by providing a low resistance pathway (Colmer, 2003). Further, many wetland

plants develop a barrier to radial O_2 loss (ROL) in the basal zones of the roots. This prevents the roots from loss of O_2 to the anaerobic soil, but also protects against intrusion of phytotoxins (Konnerup et al., 2017).

Some wetland plants produce new acclimated 'semi-aquatic' leaves. These leaves may be thin, more elongated, have reduced cuticles, few or no stomata, and chloroplasts closer to epidermis which all contribute to a lower resistance of CO_2 uptake and enhance underwater photosynthesis by optimising gas exchange (Mommer and Visser, 2005). However, some terrestrial wetland species, including rice, can photosynthesize during submergence if light and CO_2 concentrations are adequate. Underwater photosynthesis is enhanced by the superhydrophobic wax structures on leaf surfaces, which form a thin gas layer, when submerged (Raskin and Kende, 1983, Colmer and Pedersen, 2008, Pedersen et al., 2009, Winkel et al., 2013).

Leaf gas film on submerged, hydrophobic leaves

The presence of leaf gas films (LGF) provides an enlarged water-gas interface between leaves and floodwater which may avoid the problem of high cuticle resistance during submergence (Colmer and Pedersen, 2008). Therefore, stomata may remain open if gas films are present, as stomata have been suggested to close upon submergence of leaves without gas films, and gas exchange therefore occurs across the cuticle (Mommer and Visser, 2005, Colmer and Pedersen, 2008). As a result, gas diffusion is restricted by the slow molecular diffusion in the aqueous diffusive boundary layer and the resistance of the cuticle (Mommer and Visser, 2005). Pedersen et al. (2009) showed that the resistance of CO_2 uptake was reduced fivefold at environmentally relevant CO_2 concentrations by the presence of gas films in *Oryza sativa* L. Thus, gas films contribute to plant survival as it enhances the internal O_2 and sugar status by enabling underwater photosynthesis (Pedersen et al., 2009). The beneficial effect of improved gas exchange of CO_2 and O_2 due to gas films have been demonstrated for several terrestrial and wetland plants (Raskin and Kende, 1983, Colmer and Pedersen, 2008, Pedersen et al., 2009, Winkel et al., 2014, Konnerup and Pedersen, 2017, Konnerup et al., 2017, Winkel et al., 2017). However, the superhydrophobic wax structure also serves another trait for terrestrial plants, as the hydrophobic leaf surfaces facilitate water droplets to roll of and there by self-clean their leaf surfaces, as the gas exchange would be reduced on the leaf surface if covered with water (Neinhuis and Barthlott, 1997, Winkel et al., 2014).

Rice, a terrestrial wetland plant, possess thin leaf gas film on both the adaxial and abaxial side of the leaf when submerged. Winkel et al. (2014) studied the leaf gas film and retention of four genotypes of rice including FR13A, the flood tolerant donor of *SUB1A*. Underwater net photosynthesis (P_N) declined with submergence time corresponding with the loss of leaf gas film after 4-6 days of submergence. However, FR13A retained its leaf gas film for longer time than the other genotypes, but this was not related to the *SUB1A* gene. Instead, Kurokawa et al. (2018) identified *LGF1* as the gene determining leaf gas films for rice. Hence, leaf gas film presence may add to submergence tolerance for flash floods, but since the gas film disappear with time, it may not be beneficial during prolonged floods. In addition, it has been suggested that leaf gas films may protect against infections in the floodwater, as the leaf gas films form an effective barrier preventing colonization from microorganisms (Winkel et al., 2014). There are 22 wild rice species, in which leaf gas films have not yet been investigated. A screening is needed, as they may possess thicker leaf gas films, or retain their gas film for a prolonged period than common rice cultivars.

Aims and hypotheses

The objective of the present study was to investigate leaf gas film persistence during complete submergence of two wild rice species: *Oryza australiensis* and *Oryza barthii*. Additionally, underwater net photosynthesis (P_N), leaf porosity and plant length were assessed. The plants were completely submerged for a maximum of 17 days. *O. australiensis* is widely distributed in Northern Australia where it seasonally experiences dry and hot conditions (Atwell et al., 2014, Henry, 2018). *O. barthii* is widespread in West Africa and is usually found in shallow ponds with stagnant or slow flowing water (Wambugu and Henry, 2018). Based on their exposure to extreme temperatures and low moisture both species are suggested as candidates for heat and drought tolerance (Atwell et al., 2014, Menguer et al., 2017). However, both wild rice species are not well-studied, and their leaf gas film thickness have not yet been determined (Henry, 2018, Wambugu and Henry, 2018).

The following hypotheses was proposed for the study:

- Leaf gas films are expected to diminish with time during complete submergence.
- The rate of underwater P_N is expected to decrease with time during complete submergence.
- Leaf porosity is expected to diminish with time during complete submergence.
- Underwater P_N are expected to be positively correlated with thicker leaf gas films as leaf gas film improve O_2 and CO_2 exchange during submergence (Pedersen et al., 2009).

- Underwater P_N are expected to be positively correlated with a higher leaf porosity as internal transport of O_2 (and other gases) is improved by tissues with a high gas volume/tissue volume (Colmer, 2003).
- As *O. barthii* and *O. australiensis* originate from different habitats, their response in the above variables to submergence is expected to differ.

Materials and methods

Plant materials and growth conditions

Seeds of wild rice species (*O. australiensis* and *O. barthii*) provided by the International Rice Research Institute (IRRI, Philippines) were germinated following treatment to break dormancy (Timple et al., 2018). Seeds were exposed to heat treatment at 50 °C for 7 days and followed by 7 days acclimation at room temperature. Seeds were imbibed in aerated 0.5 mM $CaSO_4$ for 3 h and placed in petri dishes on tissue paper moistened with 0.001 M KNO_3. Petri dishes were covered with aluminum foil to prevent light and were left in constant temperature at 30 °C until sprouting occurred (4-7 days).

Sprouted seeds were transplanted to plastic pots with drainage holes at the bottom (diameter 100 mm, height 110 mm) with a substrate mixture 2:1 of soil (Pindstrup Substrate, Pindstrup Mosebrug A/S) and sand (0.9-1.6 mm). Pots were watered from above frequently and placed in trays with approximately 1-2 cm water. The experiment was conducted in a greenhouse with natural light, supplied with an artificial full spectrum light source (Valaya, BX120c2, Finland) for 14-16h (mean ±450 µmol photons m^{-2} s^{-1}). Temperature in the greenhouse averaged 28/22 °C Day/Night (6AM-6PM/6PM-6AM).

Experimental design

Submergence treatment commenced when plants were 5 weeks old. Each plant was considered as an experimental unit. 20 plants from *O. australiensis* and 14 plants *O. barthii* were mixed and distributed between five buckets in total (Figure ***1***). At each time point, replicates of each wild rice were harvested from each bucket; A, B, C, D and E (*O. australiensis*, n = 5, *O. barthii*, n = 3-5). As an initial control point, plants were not submerged at day 0 (*O. australiensis*, n=5, *O. barthii*, n=5). Seeds of many wild rice species exhibit strong seeds dormancy (Timple et al., 2018). Despite the above-mentioned efforts to brake seed dormancy, low germination of *O. barthii* seeds resulted in inadequate

number of *O. barthii* plants available for the submergence treatment. Therefore, the number of *O. barthii* replicates were limited on day 2, 4, 8 and 17 to 3-4 replicates compared to 5 replicates in *O. australiensis* (Figure ***1***). Fewer replicates were preferred than removing one entire time point, as this would complicate the following statistical analysis. Further, two small plants of *O. barthii* were continued growing in pots with added fertilizer (VitaGro Drivhusgødning, NPK 5-1-4, Bayer Garden, Kgs. Lyngby). These were used as initials control points when they had reached approximately same developmental stage as the initials had upon submergence.

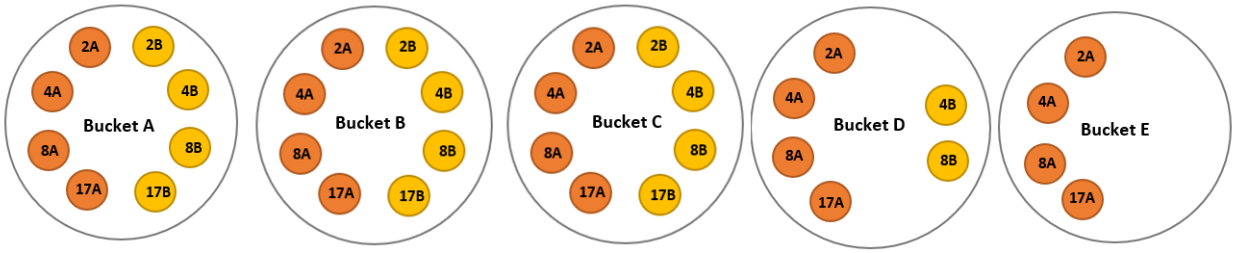

Figure 1. Experimental setup. Distribution of *O. australiensis* plants (A) and *O. barthii* plants (B). Numbers depicts the day the plant was harvested during submergence (2 = day 2, … 17 = day 17). The lacking number of *O. barthii* replicates in buckets D and E are due to low germination (65 %) of *O. barthii* seeds (see text for further explanations).

At the day of submergence, soil and sand was carefully washed from the plant root system before submergence. The plant root system was encapsulated in a plastic bag filled with sand, a metal weight, water and closed with a plastic strip. This was done to avoid soil-derived nutrients to cause algae growth in the submergence solution, and to prevent the plants from floating during submergence. Four of each wild rice plants were completely submerged in each bucket (21.7 L, diameter 29.5 cm, height 39.6 cm). Buckets were filled with tap water, and water was changed during submergence every fourth day. Measurements of leaf gas film (LGF) thickness, leaf porosity and underwater net photosynthesis (P_N) analysis were conducted on day 0, 2, 4, 8 and 17 of submergence. At each time point, the youngest fully expanded leaf at time of submergence was harvested for analysis.

Leaf gas film thickness

LGF thickness was measured following the approach of the 'buoyancy method'(Raskin and Kende, 1983, Thomson et al., 1990). Approximately 2-5 cm^2 of the middle third of the leaf was excised for measurements. The leaf segment was mounted on a hook underneath a four-digit balance and buoyancy was determined in DI water before (w_0) and after (w_1) gas film removal. Gas film was removed by gently brushing with 0.1 % (v/v) Triton X-100 on both the adaxial and abaxial side of the leaf and then rinsed thoroughly with DI water. Leaf segment areas were measured using scanned

photos and image analysis in ImageJ (Schneider et al., 2012). Leaf gas film thickness was calculated as:

$$LGF\ thickness\ (\mu m) = \frac{w_1(mm^3) - w_0(mm^3)}{2 \cdot area\ (mm^2)} \cdot 1000 \quad (1)$$

Leaf porosity

Leaf segments from LGF thickness analysis were subsequently used to determine leaf porosity. Fresh mass (FM) was recorded. Subsequently, the leaf segments were placed in a desiccator and vacuumed infiltrated with DI water at room temperature three times for five minutes. The buoyancy was then determined (w_2) and the following equation (2) was applied to determine the porosity of the leaf tissue (gas-filled volume per unit tissue volume):

$$Leaf\ porosity\ (\%) = \frac{W_1\ (g)}{W_2\ (g)} \cdot 100 \quad (2)$$

Underwater net photosynthesis (P_N)

The method for measuring underwater photosynthesis was conducted as described in Pedersen et al. (2013) which is based upon detection of O_2. Leaf segments of 1-2 cm^2, from the middle third of the leaf, were incubated in a medium with a known CO_2 concentration (see later in this section) in closed glass vials (±27 mL) with two glass beads added to ensure mixing during incubation time. Vials were filled from the bottom using a siphon to minimize exchange of O_2 and CO_2 with the atmosphere. The vials were incubated for a known time (60-150 min) using a rotating wheel submerged in a tank filled with water at a constant temperature of 25°C. Blanks, vials without leaf segment, provided the starting pO_2. During incubation sufficient photosynthetically active radiation (PAR) was provided inside the vials. A medium representing near-ambient rice floodwater with a CO_2 concentration of 200 µmol L^{-1} was mixed by following example 1, p. 12, in Pedersen et al. (2013). 1 mL $CaCl_2$, 1 mL $MgSO_4$, 2.2 mL $KHCO_3$ and 0.775 mL HCl was added to 1 L of DI water providing a solution of 200 µmol free CO_2 L^{-1}.

Dissolved O_2 concentration was measured in each vial following incubation using an O_2-optode connected to an optode meter (MicroOptode Meter; Unisense A/S). Projected areas were measured as described in previous section (LGF thickness) and net photosynthesis (P_N) was calculated as the net O_2 production by each leaf segments against blanks. The following equation (3) was used to calculate P_N:

$$P_N(\mu mol\ O_2\ m^{-2}\ s^{-1}) = \frac{\Delta O_2(\mu mol\ L^{-1}) * V_{vials}(L)}{(Time(s) * Area\ (m^2))} \quad (3)$$

Plant length

Length was measured at day 24 following submergence and compared with the length of drained control plants which had not been submerged. All plants were 8 weeks and 3 days at the day of measurements. Thus, this was done to assess if elongation was stimulated during submergence. Plant length was measured using photos with a fixed scale and image analysis in ImageJ (*O. australiensis, n=5, O. barthii, n=4-5*).

Data analysis

GraphPad Prism version 9.1.0 (GraphPad Software, La Jolla, CA, USA) was used to construct figures and to perform all statistical analyses of data. LGF thickness, leaf porosity and underwater P_N data were analysed using a two-way ANOVA test. The independent variables were time and species. Variance homogeneity was confirmed by visual inspections of residual plots, QQ-plots, and Spearman's test for heteroscedasticity. Data for P_N was log-transformed to improve variance homogeneity and normality. A one-way ANOVA was performed for plant length to test for significant differences between treatments (submerged and control). Data was checked for normality and variance homogeneity as above-mentioned for two-way ANOVA. Significance level was set to $P<0.05$ for all analyses. Subsequently, a *post hoc* Šídák's test was performed if a significance effect were found to elucidate where the effect could be found for both two-way and one-way ANOVA. Correlations between LGF thickness and P_N, and porosity and P_N was analysed using a non-parametric Spearman rank correlation coefficient test. Spearman rank correlation coefficient was chosen as a scattergram of the bivariate did not show a linear correlation (Figure ***4***) which is one of the assumptions for Pearson's product moment correlation coefficient.

Results

LGF and leaf porosity decreases with time during complete submergence

The initial mean gas film thickness was 25 µm and 24 µm for *O. australiensis* and *O. barthii*, respectively. Subsequently, LGF thickness declined during complete submergence and at day 17 the mean LGF thickness was 4 µm and 5 µm for *O. australiensis* and *O. barthii*, respectively. In Konnerup et al. (2017) the detection limit was set at 2 µm, indicating that most of the gas film had disappeared at day 17 for both wild rice species (Figure ***2****a*). A two-way ANOVA showed a significant main effect of time ($P<0.0001$), and no significant effect was found with species and with species ×

time interaction (Table ***1***). Thus, the two wild rice species lose their gas film in the same pattern during time of complete submergence.

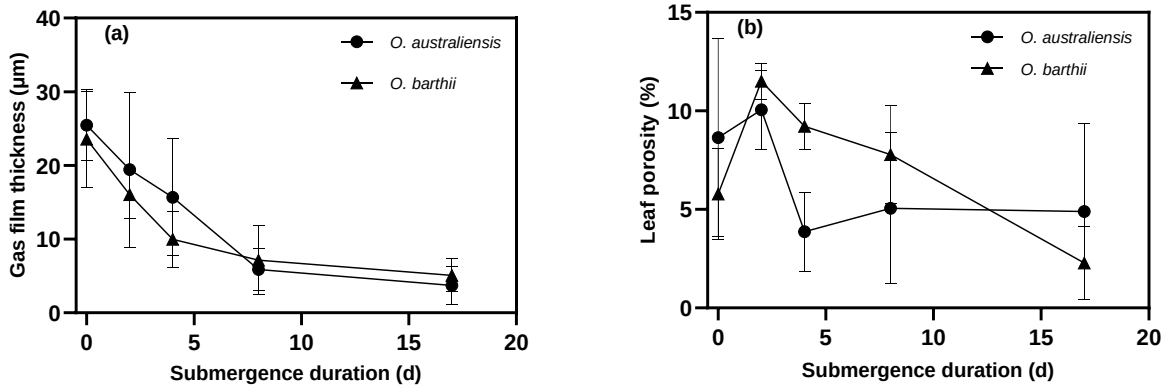

Figure 2. Leaf gas film thickness (a) and leaf porosity (b) of *O. australiensis* and *O. barthii* of the youngest fully expanded leaf with time of submergence. In (a) a two-way species × time ANOVA showed significant effect of time ($P < 0.0001$), but no significant effect was found for species or species × time (Table ***1***). In (b), two-way species × time ANOVA showed a significant time effect ($P<0.0021$) and species × time effect ($P = 0.0362$). Values are means (±SD, n =3-5).

Initially, mean leaf porosity was 8 % and 6 % for *O. australiensis* and *O. barthii*, respectively (Figure ***2****b*). At day 2, mean leaf porosity was higher in both wild rice species and was 10 % for *O. australiensis* and 12 % for *O. barthii*. At day 17, mean leaf porosity had reduced to 5 % and 2 % for *O. australiensis* and *O. barthii*, respectively. The results of a two-way ANOVA test showed a significance interaction effect with species × time (P=0.0362). A significant effect of time was also found (P=0.0021), but no significant effect for species (Table ***1***). I.e., leaf porosity in the two species did not decrease identically with time of submergence. At day 4 and 8 *O. barthii* had higher leaf porosity (9 % and 8 %) than *O. australiensis* (4 % and 5 %), which can indicate that *O. australiensis* loses its porosity faster than *O. barthii* following submergence (Figure ***2****b*).

Table 1. The effect of species (*O. australiensis* and *O. barthii*) and time of submergence (0, 2, 4, 8 or 17 days) analysed by a two-way ANOVA test. The table are showing *F*-ratios, *P*-values and the percentage of variation explained by the variables (species × time, species, and time) for LGF thickness, leaf porosity and underwater P_N. n.s. means not significant ($P > 0.05$).

Parameter	Species × Time effect			Species effect			Time effect			Data in Figure no.
	F-ratio	*P*-value	Variation %	*F*-ratio	*P*-value	Variation %	*F*-ratio	*P*-value	Variation %	
LGF thickness	0.58	n.s.	2.1	0.86	n.s.	0.8	17.63	< 0.0001	63.4	2*a*
Leaf porosity	2.9	0.0362	17.5	0.72	n.s.	1.1	5.24	0.0021	31.7	2*b*
Underwater P_N	0.17	n.s.	0.7	0.3	n.s.	0.4	14.01	< 0.0001	54	3*a*

Underwater P_N is negative after 17 days of complete submergence

At day 0, underwater P_N was 0.97 and 0.81 µmol O_2 m^{-2} s^{-1} for *O. australiensis* and *O. barthii* (Figure ***3***). The results from a two-way ANOVA showed a significant time effect (P<0.0001) (Table ***1***). Hence, P_N of both *O. australiensis* and *O. barthii* was affected by time of submergence. At day 17, mean underwater P_N decreased to -0.03 and -0.09 µmol O_2 m^{-2} s^{-1} for *O. australiensis* and *O. barthii* (Figure ***3***). This indicates that the respiration rate is higher than the photosynthetic rate, as P_N is negative.

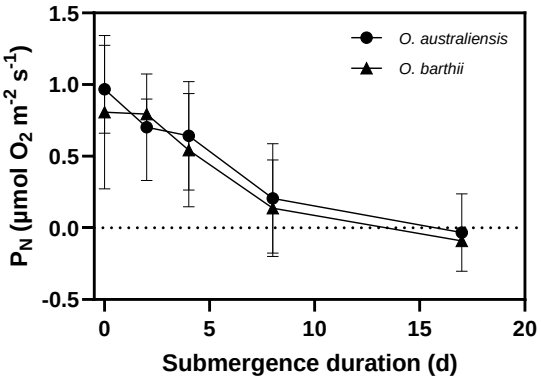

Figure 3. Underwater net photosynthesis (P_N) of the youngest fully expanded leaf at time of submergence of *O. australiensis* and *O. barthii*. A two-way ANOVA of log-transformed data showed a significant time effect (P < 0.0001). Values are means (± SD, *n* =3-5).

Underwater P_N correlates with LGF thickness and porosity

P_N was plotted against LGF thickness (Figure **3***a*) and porosity (Figure **3***b*) to test if correlation was significant. A Spearman rank correlation test revealed a significant positive correlation (r=0.6280) between underwater P_N and LGF thickness. This indicates a positive effect of LGF thickness on underwater P_N. Another Spearman rank correlation test showed likewise a significant positive correlation (r=0.4057) between underwater P_N and porosity. This indicate that higher leaf porosity adds as another positive effect on underwater P_N. However, the points with low LGF and porosity had been submerged for a longer period as leaves were harvested at day 0, 2, 4, 8 and 17, and other factors e.g., chlorophyll degradation may affect underwater P_N.

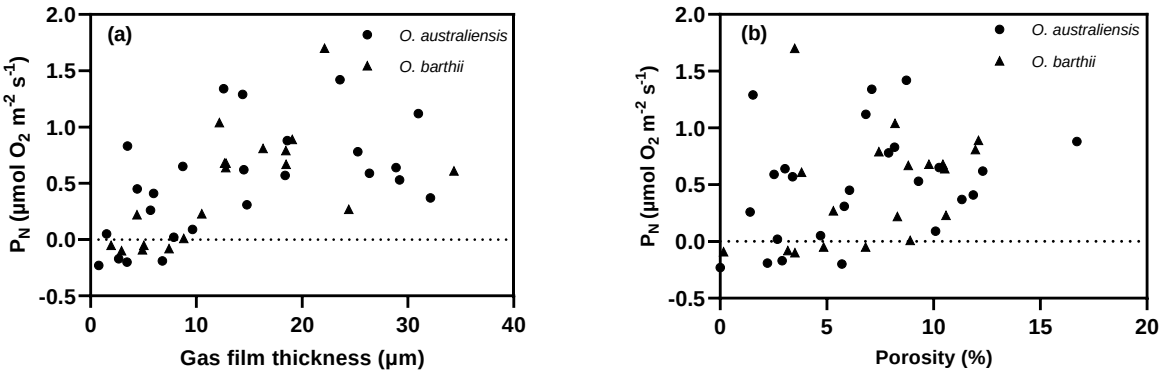

Figure 4. Underwater P_N plotted against corresponding leaf gas film thickness (a) and leaf porosity (b) for both *O. australiensis* and *O. barthii*. In (a) and (b), a non-parametric Spearman rank correlation analysis showed significant positive correlation for LGF (P <0.0001, r= 0.6280) and for leaf porosity (P=0.0063, r=0.4057). The points represent actual measurements (n=44).

O. australiensis ceased to elongate during complete submergence

A one-way ANOVA revealed a significant difference between treatments (P<0.0001). By comparing the length of submerged *O. australiensis* and *O. barthii* with their respective drained controls, a *post-hoc* Šídák's test showed a significant difference between *O. australiensis* control and submerged (P>0.0001). Mean length for *O. australiensis* submerged was 64% of the drained controls (Figure ***5***). Thus, *O. australiensis* ceased to elongate when completely submerged. In contrast, no significant difference was found between *O. barthii* submerged and control (P=0.9311) and may suggest that elongation of *O. barthii* continued at a similar rate as in air. Drained controls of *O. barthii* were very small at the start of submergence and hence, were not chosen for submergence.

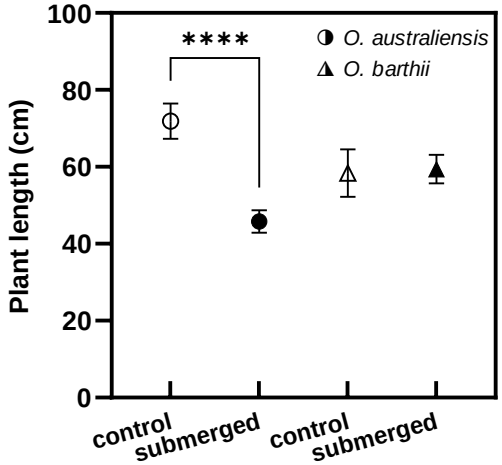

Figure 5. Plant length of *O. australiensis* and *O. barthii* plants after 24 days of submergence and control plants that had not been submerged (control and submerged plants were 8 weeks and 3 days old when measured). A one-way ANOVA test showed a significant difference between the treatments (P<0.0001). A *post-hoc* Šídák's test revealed the significant difference between '*O. australiensis* submerged' and '*O. australiensis* control' (P<0.0001). **** denotes a significant difference of P<0.0001 was found after a *post-hoc* Šídák's test. Values are means (± SD, n=4-5).

Discussion

This study demonstrated that time of submergence for *O. australiensis* and *O. barthii* had a strong negative effect on LGF thickness, leaf porosity and underwater P_N. This underlines that completely submergence exerts high stress on both *O. australiensis* and *O. barthii* with possible huge impacts on plant yield and/or survival. The results were in accordance with the proposed hypotheses of LGF, leaf porosity and underwater P_N decreasing with time of submergence. Furthermore, underwater P_N was positively correlated with both thicker LGF and higher leaf porosity. However, *O. barthii* and *O. australiensis* did not respond differently to submergence despite originating from differing habitats. In the following sections I will discuss these findings in relation to previous results.

Submergence induces leaf senescence in both *O. australiensis* and *O. barthii*

Underwater P_N significantly correlated with both LGF thickness and leaf porosity with positive correlation coefficients. However, these positive correlations do not imply a causation. Partly because the points with thin LGF and low porosity had been submerged for a longer period, and partly because we must account for other parameters in the photosynthetic apparatus which may affect underwater P_N (Winkel et al., 2014). Nevertheless, Herzog et al. (2018) also found a positive correlation between LGF thickness and underwater P_N, indicating the positive effect of LGF on underwater P_N. However, Winkel et al. (2014) reported no significant correlation between LGF and underwater P_N for four rice genotypes. Instead, they found a positive correlation between chlorophyll concentration (not studied here) and underwater P_N, suggesting that chlorophyll concentration also contribute to the capacity for underwater photosynthesis. Although, chlorophyll senescence was not measured in the present study, I observed, that during submergence, leaves of both species started to show leaf senescence which is a common feature of submerged rice, as ethylene accumulation causes chlorophyll degradation. This was especially pronounced from day 8 (Supplementary Fig. 1) (Jackson et al., 1987, Herzog et al., 2018). Additionally, leaf porosity had also declined during time of submergence, thus gas-filled tissue was infiltrated by water, and did not facilitate internal gas transport any longer. This was also evident in Winkel et al. (2014).

Underwater P_N decreases as expected, but from a surprisingly low level in both *O. barthii* and *O. australiensis*

The underwater P_N for both wild rice species are surprisingly low compared with other studies of rice which measured underwater P_N at near ambient CO_2 levels (200 µmol L^{-1}) as in this study (Winkel et

al., 2014, Herzog et al., 2018). Winkel et al. (2014) measured initial underwater P_N in four rice genotypes between 3.6-4.8 μmol O_2 m^{-2} s^{-1}. Herzog et al. (2018) measured initial underwater P_N for *Oryza sativa* L. to app. 8 μmol O_2 m^{-2} s^{-1}. In this study initial underwater P_N was only between 0.81-0.97 μmol O_2 m^{-2} s^{-1}. The underwater P_N is app. four-five times less than found in Winkel et al. (2014) and 8 times less than found in Herzog et al. (2018). However, the low rates of P_N found in this study could indicate that plants have been stressed prior to submergence or that the conditions of measurements have been different. As described in the Materials and Methods sections, prior to submergence and drained controls, plants were watered frequently, and pots were placed in 1-2 cm of water to avoid any drought stress. Light was provided for a 16 h period a day, the soil was pre-fertilized for several weeks of growth and there were no sign of fungal infections or aphids. Plants were grown in a warmed glass house to keep temperatures >20℃ during night. However, three days of the datalogger revealed that night temperature had been <20℃, and some day-time temperatures reached 45℃ due to a malfunctioning glass house ventilation system. I am not aware of any studies determining an optimal growth temperature for *O. australiensis* or *O. barthii*, but *O. sativa* is known to show decreased growth and yield when temperatures increase >32℃ (i.e., 37℃ in (Kilasi et al., 2018) and 38℃ in (Aghamolki et al., 2014)).

Additionally, in previous underwater photosynthesis measurements, leaf segments were incubated at 30℃ compared to 25℃ in the present study. The increasement of 5℃ may explain why the photosynthetic rate is higher in previous studies of rice due to the Q10 principle. However, an increasement of 10℃ typically results in rates two-three times higher (Rasmusson et al., 2019). Low PAR radiation during incubation and CO_2 levels <200 μmol L^{-1} may also have contributed to lower underwater P_N rates, as underwater P_N is strongly dependent on light availability and free CO_2 in the incubation medium (Winkel et al., 2013). Thus, a saturation curve is observed of underwater P_N for plants with leaf gas films with increasing CO_2 concentration, and without presence of gas film a linear curve is observed (Pedersen et al., 2009, Winkel et al., 2013).

Even though, the underwater P_N for this study was very low, the overall response to submergence was in accordance with other similar studies of rice (Pedersen et al., 2009, Winkel et al., 2014, Herzog et al., 2018). Thus, underwater P_N decreases with time of submergence and >8 d upon submergence underwater P_N was negative. The internal O_2 and carbohydrate levels are soon to cease due to a

negative P_N, as the rate of respiration exceeds the rate of photosynthesis (net O_2 consumption), having detrimental consequences on plant survival (Pedersen et al., 2009).

O. barthii and *O. australiensis* gas films are thinner, but retained longer than *O. sativa*

The LGF thickness found in similar studies for five different rice genotypes was almost double (50-62 μm) the LGF thickness found in this study (24-25 μm) (Pedersen et al., 2009, Winkel et al., 2013, Winkel et al., 2014). However, Herzog et al. (2018) measured LGF thickness in *O. sativa* L. var. Amaroo (similar to Pedersen et al. (2009)) and found the initial LGF thickness to be 25 μm. This variation was explained by a 10 ℃ temperature difference during measurements, or measurements on different leaf sections. Thus, this study suggests that *O. australiensis* and *O. barthii* may form thinner LGF than the five previously studied rice genotypes (Pedersen et al., 2009, Winkel et al., 2013, Winkel et al., 2014). However, an unpublished data (Kjær, J.E. 2021, unpublished) had previously investigated LGF thickness of both *O. australiensis* and *O. barthii* and reported LGF thickness of 10 μm and 30 μm, respectively. Hence, suggesting an even thinner LGF for *O. australiensis* than in this study. In order to assess the reasons behind the thinner gas film formation, future studies could measure leaf contact angle (Konnerup and Pedersen, 2017) to quantify leaf surface hydrophobicity. As *O. australiensis* and *O. barthii* are suggested candidates for heat and drought tolerance (Atwell et al., 2014), this may explain why they form a thinner LGF than the previously studied rice cultivars. Thus, investment of flood tolerant epicuticular wax structures, which form a thick LGF may not have been of competitive advantage in their originating habitat.

Gas films persisted on both wild rice species for more than 17 d of submergence (day 17: *O. australiensis* ~ 4 μm and *O. barthii* ~ 5 μm). However, underwater P_N was negative on day 17 and at day 8 it was close to zero. There might be a minimal thickness of LGF in which the improved gas exchange is no longer beneficial. However, at LGF thickness >10 μm (day 4) for both species, a positive P_N was recorded. In other studies of rice, LGF persistence have been reported for 4-6 days (Winkel et al., 2014) and >9 days (Herzog et al., 2018). Thus, in this study, both rice species retain their LGF for a longer period than previous studies. However, the loss of LGF are also dependent on floodwater conditions, and turbid floodwaters accelerates gas film loss (Das et al., 2009). In the present study, plants were submerged in tap water free from particles, while plants in Winkel et al. (2014) were submerged in turbid water under field conditions.

LGF loss has been related to the loss of leaf surface hydrophobicity. The formation of abundant epicuticular wax platelets on leaves of rice strongly affects surface hydrophobicity, gas film retention and underwater photosynthesis during submergence (Kurokawa et al., 2018). Furthermore, it has been reported that warmer temperature, lower intensity of light and turbid floodwater accelerates plant mortality (Das et al., 2009), suggesting that flood tolerance is a complex, and plant survival is highly dependent on floodwater characteristics e.g, water depth, temperature, duration, light availability and turbidity (Das et al., 2009).

Both wild rice species seems to exhibit a "quiescence response" to complete submergence similar to the *SUB1A* gene

Following submergence, plant length measurements showed that plants of *O. australiensis* ceased to elongate compared with drained control plants which may suggest that *O. australiensis* tried to avoid energy consumption associated with the quiescent strategy (Hattori et al., 2011). For *O. barthii* no significant difference was found, suggesting that elongation in submerged plants occurred at same rates as in air. However, due to the inherently strong seed dormancy in wild rice species (Timple et al., 2018), drained control plants of *O. barthii* smaller than other *O. barthii* plants, at the day of submergence, had to be included in the study in order to compensate for a low germination rate. To allow these plants to reach same developmental stage as larger plants, fertilizer was added to facilitate plant growth for these small plants. Thus, this may have had an influence on the plant length of the drained controls for *O. barthii*. Plants were not prevented from establishing atmospheric contact during submergence in the 40 cm high buckets, by using netting or similar as in Herzog et al. (2018). Although, no leaves were emergent at the start of the experiment, at day 8 I observed, 13 out of 14 *O. barthii* plants having an emergent leaf. This may suggest that *O. barthii* stops elongating after having established atmospheric contact with one emergent leaf. It has been reported that some deepwater rice can elongate 20-25 cm d^{-1} and reach a height of 7 m (Hattori et al., 2011). However, this was not even the case for *O. barthii*. Thus, both wild rice species seems to exhibit a "quiescence response" which is the same for the *SUB1A* gene. A quiescent strategy can be advantageous during a transient flash flood, as availability of carbohydrates are of importance when water subsides. However, during long periods of stagnant flooding, an escape strategy of rapid stem elongation would be more beneficial (Hattori et al., 2011).

Surprisingly, *O. australiensis* and *O. barthii* responded very similar to the submergence treatment, despite their distinct originations. I.e., LGF, porosity and underwater PN both showed no significant species effect in two-way ANOVA. However, to fully assess species submergence tolerance, a recovery period following submergence and biomass, survival and shoot carbohydrate levels would have to be monitored.

Conclusion and outlook

In brief, this study suggests that *O. australiensis* and *O. barthii* possess thinner LGF than the previous studied rice cultivars: FR13A, Swarna-Sub1, Swarna, IR42, *O. sativa* L. var. Amaroo (Pedersen et al., 2009, Winkel et al., 2013, Winkel et al., 2014, Herzog et al., 2018). There was no difference in the LGF thickness or persistence of the two species despite their different originating habitats. However, submergence time had an overall negative effect on LGF thickness, leaf porosity and underwater P_N. *O. australiensis* ceased to elongate where *O. barthii* elongated at same rate as drained control plants. The low submergence tolerance based on leaf senescence may be due to the habitat conditions, which for *O. australiensis* are most hot and dry. Thus, the formation of a thick LGF as a flood tolerant trait may not have been advantageous. This study only investigated 2 wild rice species out of 22. Hence, the LGF thickness and persistence remain unknown for the remaining 20 wild rice species. The screening of other wild rice species and their LGF thickness and persistence may be helpful in improving flood tolerance in rice. Further studies must seek to investigate the factor implicated in degradation of leaf gas film during submergence (Winkel et al., 2014).

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Supplementary figures

Fig 1: Leaf segments of gas film and porosity measurements after 8 days of submergence.