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Bachelorprojekt

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Submergence tolerance in rice cultivars

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Abstract

Rice is a global important crop threatened by climate change and the accompanied abiotic stresses. Rice fields are especially exposed to floods that has severe impact on the crop yield. Several traits beneficial towards submergence has been recognized and some genotypes have higher submergence tolerance, like those possessing SUB1 genes. One of these traits is leaf gas film, which improve the gas exchange between the leaf and its submerged environment. In this project submergence tolerance of the three cultivars of *O. sativa* (IRRI154 and FL478) and *Oryza glaberimma x Oryza sativa* (Nerica-4) were assessed and investigated through measurements of underwater net photosynthesis, leaf gas film thickness, leaf tissue porosity and leaf chlorophyll concentration. The cultivars showed low underwater net photosynthesis rates, probably due to incubation conditions. The *Oryza glaberimma x Oryza sativa* Nerica-4 had thinner gas film, however, did not otherwise markedly differ from IRRI154 and FL478. The three cultivars IRRI154, FL478 and Nerica-4 did not show markedly more submergence tolerance compared with other *O. sativa* genotypes.

Introduction

Climate Change requires rice tolerance toward abiotic stress

Climate change is changing our world. Climate change and global warming increases the sea level, flood hazards and leads to heavier precipitation (IPCC, 2021). These changes will decrease net production of crops, and this impacts food availability and security as well as economic growth, especially in low- and middle-income countries and regions (IPCC, 2021). Rice is one of the crops that is severely affected by climate change (Dar *et al*., 2021), making the vulnerability of rice towards consequences of climate change and the accompanied abiotic stresses a global problem. Paddy rice is amongst the 9th most produced commodities in the world (averagely between 1994-2019) (FAO, 2021). 90.6% of the rice is produced in Asia and 3.5% in Africa (on average between 1994-2019) (FAO, 2021). I.e., rice is the main source for calories for more than half of the population of the world (Dar *et al.,* 2021). The countries and the areas where the rice is produced are some of those most severely impacted by climate change and many here depend on agriculture and food security (FAO, 2017). The rice production has increasingly been exposed to abiotic stresses like salinity, drought and flooding causing huge losses (Dar *et al.,* 2021).

Flooding is one of the most destructive abiotic stresses for rice fields, and more than 16% of the worlds rice crop fields are exposed to flooding (Dar *et al.,* 2021). Climate change is expected to

increase flooding, irregular rainfall patterns and frequency and intensity of extreme weather events, and this is a threat to the sustainable rice production (Dar *et al*., 2021). Huge rainfed lowland areas of tropical Africa and South and Southeast Asia are affected by flash floods, which is complete submergence for 10-15 days (Winkel *et al*., 2012), which decrease the rice field productivity (Das *et al*., 2009). These areas can especially be affected due to poor or not existing drainage and the position of the areas prevent fast water movement away from the fields (Ismail *et al*., 2013).

The development and incorporation of submergence tolerance in rice cultivars could ease the consequences of flooding and climate change's effect on rice production (Dar *et al*., 2021). To reach this goal, an understanding of the effects of floods on plants and plants response is needed.

Flooding and effects on plants

A flooding event is typically categorized into two levels; waterlogging and submergence (Jia *et al*., 2021). Waterlogging is when the roots are surrounded by water (Colmer & Voesenek, 2009). During waterlogging the soil become anoxic, potential toxic compounds such as Fe^{2+}, Mn^{2+}, S^{2-} and carboxylic acid with phytotoxic effects on the roots can accumulate and the nutrient uptake can be decreased, which reduces the plant growth (Konnerup *et al*., 2017). When submergence occurs, the whole plant is partially or completely submerged in water (Colmer & Voesnek, 2009). During submergence, the roots are waterlogged, and the shoots are submerged, and it is therefore a more severe type of flooding (Colmer & Voesenek, 2009).

Submergence affects plants in many ways. It challenges the plants gas exchange; gasses diffuse 10.000 times slower in water than in air (Colmer & Voesenek, 2009). The severely inhibited gas diffusion impacts the availability of CO_2 for photosynthesis and O_2 for aerobic respiration (Colmer *et al*., 2011). The plant can therefore experience an energy crisis due to lack of O_2, as the cells become anoxic, the oxidative phosphorylation ceases, halting ATP production (Colmer & Voesenek, 2009). ATP can be produced through glycolysis, if NAD^+ is regenerated, but with a lower output then produced through oxidative phosphorylation. Small amounts of ATP lead to impairment of the cell and its components (Colmer & Voesenek, 2009). Limitation of CO_2 uptake can lead to a carbohydrate crisis and without soluble sugars and starch the plant cannot sustain respiration or glycolysis and the ATP production. Deficiency of sugar and starch will ultimately lead to cell death (Colmer &Voesenek, 2009).

The synthesis of the plant hormone ethylene is promoted by the low O_2 concentrations and high CO_2 concentrations occurring during flooding and accumulates in the submerged tissue due to slow

outward diffusion (Mori *et al*., 2019). Ethylene promotes shoot elongation (observed in rice) which in some scenarios can lead to depletion of carbohydrate and therefore increased mortality (Das *et al.,* 2009). Plants can also experience light limitation during submergence if the floodwater is turbid or deep. This will affect the photosynthesis and the plants carbohydrate reserve (Pedersen *et al*., 2009). The effects of submergence on the plants are complex and the degree of damage derived by the submergence depends on the duration, depth, and condition of the floodwater and the topography of the flooded area (Konnerup *et al*., 2017).

Several traits are linked to increased flood tolerance, such as internal aeration, maintaining the reserves of carbohydrate, reducing or increasing shoot elongation, preserving chlorophyll, and minimizing post-submergence oxidative stress (Winkel *et al*., 2014) (Ismail, Singh *et al*., 2013) as described in the following paragraphs.

Internal gas transport: Root aerenchyma and ROL

One of the traits linked to flood tolerance is optimizing the internal gas transport. During submergence roots cannot absorb O_2 from the anoxic soil. A trait enabling root O_2 supply is formation of root aerenchyma. Root aerenchyma allow internal O_2 and CO_2 movement from shoot tissue to root extremities through interconnected gas-filled spaces (Colmer & Voesenek, 2009). Thus, root aerenchyma provide a low resistance pathway for the gasses from the shoots to the roots. Another trait is radial O_2 loss (ROL) barrier created by the roots. The barrier prevents O_2 loss to the anoxic waterlogged soils so the root tips can use the O_2 for growth and limit phytotoxin entry (Colmer, 2003). These traits are seen in wetland plants including rice (Colmer, 2003).

Strategies against submergence

Terrestrial wetland plants have evolved two different strategies for survival during submergence: Low Oxygen Quiescence Syndrome (LOQS) and Low Oxygen Escape Syndrome (LOES) (Colmer and Voesnek, 2009). With the LOQS strategy the shoots do not elongate and in some scenarios cease all growth for conserving substrates and prolonging survival during submergence. This happens through economic use of ATP, increase of enzymes for production of ATP without molecular O_2 and increase in production of components that inhibits the cellular changes during flooding. With the LOES strategy the plant elongates above water by redirecting growth and increases growth in shoots, develop or retain physical structures that facilitate internal gas diffusion and develop or preserve structures available of exchange of gas between the plant and the submerged environment (Colmer & Voesnek, 2009). During slow rising floods elongation and

LOES is a successful strategy, but with flash floods LOQS is an advantage (Colmer & Voesnek, 2009). The two strategies differentiate in their ethylene responsive factors encoded by the SUB1 locus (Bailey-Serres *et al.,* 2010). SUB1 is a quantitative trait locus (QTL), associated with submergence tolerance, encoding three ethylene responses (Winkel *et al.,* 2014). These responses control traits as limitation of shoot elongation, maintenance of soluble carbohydrate reserve, less chlorophyll degradation and less oxidative stress post-submergence. Traits all contributing to submergence tolerance (Winkel *et al.,* 2014). SUB1 has been transferred into numerous varieties, the varieties with the SUB1 genes showed same yields as their counterparts whilst showing better yields (1 t ha^{-1} to more than 3 t ha^{-1}) after complete submergence in fields of various duration (Winkel *et al*., 2014).

A trait enabling significant underwater photosynthesis: leaf gas-films

During submergence some terrestrial wetland plants, including rice, can facilitate underwater photosynthesis and produce carbohydrate (Winkel *et al.,* 2014). Some plants have superhydrophobic leaves, which is considered as a self-cleansing mechanism (Winkel *et al.,* 2016), resulting in the formation of a thin gas film surrounding submerged, superhydrophobic leaves. Wetland plants retaining a leaf gas film upon submergence are capable of continuing the photosynthesis underwater. The gas film enlarges the diffusion interface between the plant tissue and the submerged environment (Colmer *et al.,* 2011), enabling plants with superhydrophobic leaves to keep relatively high underwater photosynthesis rates during the day and improve intake of O_2 during the night (Winkel *et al.,* 2017; Konnerup *et al.,* 2017). Furthermore, it has been shown in submerged terrestrial plants that leaf gas film has a positive impact on internal aeration of root tissue, sugar levels and growth during complete submergence due to the underwater photosynthesis (Pedersen *et al.,* 2009). Leaf gas films also impact leaf tissue chlorophyll and porosity (Winkel *et al.,* 2017), both declines faster during submergence in plants with no gas film (Herzog *et al.*, 2018).

However, the leaf gas film degrades during submergence (Konnerup *et al.,* 2017) and disappears after 1-11 days of submergence (Winkel *et al.,* 2016). Leaf gas film persistence depends on leaf hydrophobicity (Konnerup *et al.,* 2017). Hydrophobicity, and thereby the leaf gas film retention time, is related to the wax platelets on the cuticle (Konnerup *et al.,* 2017). However, the exact mechanism behind cuticle deterioration and leaf gas film collapse remains to be established. *Winkel et al*., (2014) showed, that leaf gas film persistence differed between rice cultivars in a field situation. Meanwhile, wheat cultivars did not seem to differ in leaf gas film persistence when submerged under controlled conditions (Konnerup *et al*., 2017). Floodwater composition also seems

to affect leaf gas film persistence, as it has also been shown that leaf gas film has longer preservation in non-saline water compared with saline water (Herzog *et al.*, 2018).

Other rice species than *Oryza sativa* have been suggested to serve as donors for flood tolerance traits (Attwell *et al*., 2014), warranting the investigation of flood tolerance in other species such as *Oryza glaberrima.*

Oryza sativa and Oryza glaberrima

O. sativa (Asian rice) and *O. glaberrima* (African rice) are two cultivars belonging to the Oryza genus (Nayar, 2014). The Oryza species are pantropical and grow in many different habitats but are primarily adapted to seasonal wetlands and wetlands (Nayar, 2014) (Vaughan *et al.,* 2003). Genotypes of *O. sativa* gives a better yield than *O. glaberimma* and are therefore cultivated in more areas. (Sakagami, 2012). However, *O. glaberrima* grows adequately in environments exposed to abiotic stresses, like flooding (Sakagami, 2012).

Aim and Hypotheses

The aim of this project was to investigate submergence tolerance of three rice cultivars of the species *O. sativa* and *O. glaberrima* through measurements of underwater net photosynthesis, leaf gas film, leaf porosity and leaf chlorophyll with time of submergence. The study was testing the following hypotheses:

- Nerica-4 will have a different response to submergence compared to the two *O. sativa* cultivars, since Nerica-4 is a *O. sativa* × *O. glaberrima* crossing.
- Leaf gas film thickness will decrease with time of submergence.
- As the gas film decreases, the leaves will experience senescence and the tissue porosity and chlorophyll concentration will decrease, all of which will cause a decline in underwater net photosynthesis.

Methods

In this project three rice cultivars were assessed: *Oryza glaberimma x Oryza sativa* 'Nerica-4' from Sokoine University of Agriculture (Tanzania), *Oryza sativa* salt tolerant 'FL478' and *Oryza sativa* salt intolerant 'IRRI154' from International Rice Research Institute (Philippines) (Table 1).

Table 1: Rice cultivars

Cultivar	Abbreviation	Ability
Oryza glaberimma x Oryza sativa	Nerica-4	No knowledge of salt tolerance or intolerance
Oryza sativa	FL478	Salt tolerant
Oryza sativa	IRRI154	Salt intolerant

The rice seeds were germinated, planted, and then submerged for underwater net photosynthesis, gas film, porosity, and chlorophyll measurements.

Plant culture

To start the germination the rice seeds imbibed in an aerated 0.5 mM $CaSO_4$ solution for 3 hours. The solution was covered in aluminum-foil to keep a dark imbibition. The seeds from the three cultivars were placed in petri-dishes with paper towel moistened by 0.5 mM $CaSO_4$ solution and covered in aluminum-foil. The FL478 salt tolerant and IRRI154 salt intolerant seeds germinated for 3 days and the *O. glaberimma x O. sativa* germinated for 4 days in a climate room at 30°C. Following germination, seedlings were planted in 2 parts soil (Pindstrup Substrate, Pindstrup Mosebrug A/S) and 1 part sand (0.9-1.6 mm) in plastic pots (height 11 cm, width 10 cm) with drainage holes. After growing for 27-28 days the plants were submerged in tap water for 17 days in 5 plastic buckets. The measurements were conducted at five timepoints (day 0, 2, 4, 8 and 17). For each timepoint there were 5 replicates from each cultivar (except day 17 there were 4 replicants of *O. glaberimma x O. sativa*), in total 74 plants. The replicates were assigned to the buckets so that replicate 1 from each cultivar and timepoint were in the same bucket, replicate 2 from each cultivar and timepoint were in the same bucket and so forth. Due to an unexpected low germination rate of NERICA-4 seeds, and therefore lack of plant material for measurements, five small plants had to be kept at drained conditions for several days until day 0 measurements. Thus, the NERICA-4 day 0 measurements were conducted 17 days after the submergence treatment commenced. During this period, drained plants were fertilized with liquid fertilizer (VitaGro, Bayer Garden) two times.

During the growing and submergence period, the plants had following conditions concerning light and temperature: at daytime on a partly cloudy day, the average of light in the air was 450.2 μmol photons m^{-2} s^{-1} and 341.1 μmol photons m^{-2} s^{-1} in the water. The temperature from 1st of March to 15th of April 2021 was between 6am and 6pm max. 41.51 °C, min. 16.28 °C and averagely 28.27 °C. Between 6pm and 6am it was max. 30.20 °C, min. 15.99 °C and averagely 22.35 °C.

Underwater net photosynthesis

Underwater net photosynthesis was measured as net O_2 production by leaf segments following the method described by *Pedersen* (2013). Leaf segments were incubated in transparent vials with a defined medium (described below) for a known amount of time (100-250 minutes). The vials contained two glass beads and were rotating on a vertical wheel (custom-made incubator for underwater photosynthesis) to ensure mixing. They were incubated at a specific temperature (25 °C) and light intensity (500 μmol photons m^{-2} s^{-1}). The medium consisted of 1-liter demineralized water, 1 ml $CaCl_2$, 1 ml $MgSO_4$, 2.2 ml $KHCO_3$ and 0.775 ml HCl, rendering a final concentration of free CO_2 of 200 μM (Mackereth *et al.,* 1979). For each timepoint (day 0, 2, 4, 8 and 17) leaf segments from five replicants from each cultivar were measured. The leaf segment of around 1-2 cm^2 was excised from the middle third of the youngest fully expanded leaf at time of submergence (leaf tips were excised from the youngest fully expanded leaf at time of submergence in order to identify the target leaves during the submergence treatment). Leaf segments were kept in a weigh boat with water from the allocated bucket before placing them into the medium filled vials. After incubation, the dissolved O_2 concentrations were measured in the vials with an O_2 optode (Pyroscience OXROB3) connected to a computer and measured with the program Unisense logger. The O_2 optode were calibrated in 25 °C water at air equilibrium (20.6 kPa O_2) and in anoxic water (0.0 kPa O_2) containing 100 mM NaOH and 100 mM sodium ascorbate (Herzog *et al.*, 2018). Projected area of leaf segments was measured by scanning the leaves and analyzing in the program ImageJ (Schneider, 2012). The leaf segments net O_2 production was measured against blanks: vials filled with the same medium without leaf segments. The underwater net photosynthesis was then calculated as μmol O_2 m^{-2} s^{-1}.

Gas Film

Leaf gas film thickness was measured using the 'buoyancy' method by *Raskin* (1983). For each timepoint (day 0, 2, 4, 8 and 17) leaf segments from five replicants from each cultivar were measured. Leaf segments around 5-10 cm^2 were cut of the middle third of the youngest fully

developed leaf and placed in weigh boats with demineralized water. The leaf segments buoyancy was determined before and after gas film removal. The gas film was removed by gently brushing the leaf segments on both sides with Triton X-100 (0,1%). The projected leaf area was determined as described earlier (Underwater net photosynthesis Methods). The gas film was then calculated by following equation:

$$GF(\mu m) = \frac{w_1 - w_0}{2 \ times \ projected \ area \ (cm^2)} * 10.000$$

w_1 = leaf weight after gas film removal (g)

w_0 = leaf weight before gas film removal (g)

Porosity

Porosity of the leaves was, as gas film, measured using the 'buoyancy' method according to *Raskin* (1983). For measurement of porosity the leaf segments with removed gas film were used. The fresh mass of the leaf segments was measured by placing segments on a 4-digit balance. Segments were then vacuum infiltrated by placing them in a beaker filled with DI water inside a desiccator connected to a vacuum pump. Vacuum as applied three times, 5 minutes duration at -1 bar. Post vacuum the leaf segments were measured again according to the 'buoyancy' method (Raskin, 1983). The porosity was calculated in % by following equation:

$$Porosity \ \% = \frac{Vol. \ gas \ leaf * 100}{Vol. \ leaf}$$

After the measurements, the leaf segments were stored in a freezer for chlorophyll measurements.

Chlorophyll

The leaf segments used for gas film and porosity measurements were freeze dried for 24 hours at 1 mbar vacuum. The leaf segments were then homogenized in Eppendorf tubes with two steal-beads in a mini-bead beater for 10 sec. Dry weight was measured, and the chlorophyll was extracted in 96% ethanol wrapped in aluminum-foil for 7 days at 5 °C. The leaf segments were then centrifuged (KUBOTA KS-8000) for 3 min at 3000 rpm and the chlorophyll-a absorbance of the supernatant measured at 665 nm and 750 nm at a spectrophotometer (UV-1800, Shimadzu). The chlorophyll concentration calculation was based on *Mackinney* (1941) conferring to *Herzog et al.* (2018) and *Konnerup et al.* (2017). Chlorophyll was calculated as µg Chlorophyll-a mg^{-1} dry weight.

Statistics

Data was analyzed using GraphPad Prism version 9.1.0, 2021, (GraphPad Software, San Diego, CA, USA). Two-way ANOVA was used to analyze underwater net photosynthesis, gas film, porosity, and chlorophyll. None of the datasets passed the normality tests in the two-way ANOVA analysis. Transformation (Y=Log(Y)+1) of data was performed to achieve a more Gaussian distribution (GraphPad, 2021) and to obtain better residual and QQ plots. When the data set from underwater net photosynthesis was transformed the Kolmogorov-Smirnov test for normality was passed, the residual and homoscedasticity plot were more randomly spread and the QQ plot were more linear. The gas film dataset passed two normality tests, D'Agostino-Pearson omnibus, and Shapiro-Wilk, when transformed and the plots had a better tendency. When transformed, the dataset for porosity passed the normality test Anderson-Darling. The Homoscedasticity plot were more randomly spread and the QQ plot more linear. The data set from chlorophyll log-transformed also showed better plots. Due to the equal sample sizes and ANOVA's robustness towards violations of a Gaussian distribution in analysis with large samples (GraphPad, 2021) the test was deemed a suitable model to analyze the data. The two-way ANOVA was followed up by a multiple comparisons Tukey's test. Correlation between underwater net photosynthesis and gas film, porosity and chlorophyll were assessed with a nonparametric Spearman correlation with a two-tailed P-value.

Results

Decline in underwater net photosynthesis is similar in all cultivars

Underwater net photosynthesis was measured in the three rice cultivars during 17 days of submergence. In all three cultivars, the rate of underwater net photosynthesis (P_N) decreased with time of submergence (Figure 1). Nerica-4, FL478 and IRRI154 had a P_N rate at respectively 1.74 µmol O_2 m^{-2} s^{-1}, 0.97 µmol O_2 m^{-2} s^{-1} and 0.96 µmol O_2 m^{-2} s^{-1}, at day 0 and ended at -0.08 µmol O_2 m^{-2} s^{-1}, -0.07 µmol O_2 m^{-2} s^{-1} and -0.03 µmol O_2 m^{-2} s^{-1}, at day 17. The P_N rates for IRRI154, FL478 and Nerica-4 ended in negative values which means the leaves used more O_2 than they produced, and they did more respiration than photosynthesis.
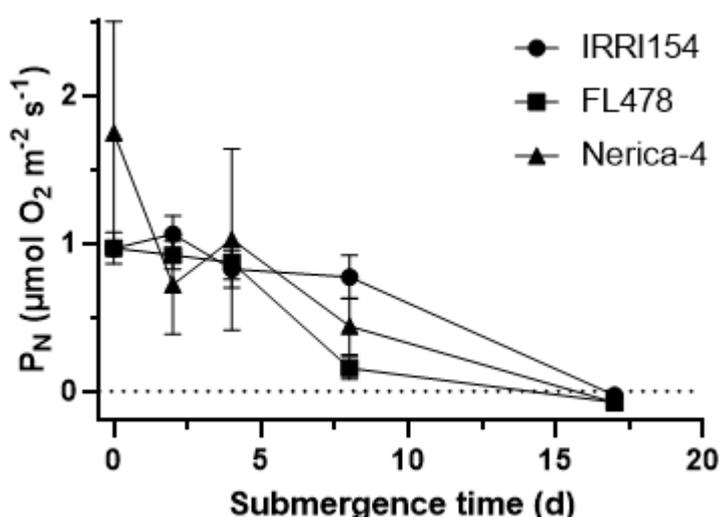

Figure 1: Underwater net photosynthesis of leaf segments of *O. glaberimma x O. sativa* 'Nerica-4', *O. sativa* salt tolerant 'FL478' and *O. sativa* salt intolerant 'IRRI154' with time of submergence. Symbols represents means ± SEM, n=4-5.

Compared to IRRI154 and FL478, Nerica-4 had an 81.3% and 79.4% higher rate of P_N before submergence (day 0), respectively (Figure 1). IRRI154 maintained P_N at initial levels until day 8, while P_N in FL478 and Nerica-4 had already decreased to 15.5% and 25.3% of initial values by day 8 (timepoint 4) (Figure 1). At day 8 the post-hoc Tukey test showed significant differences between IRRI154 and respectively FL478 (P=0.0033) and Nerica-4 (P=0.0119). The three cultivars were clearly affected by submergence. The P_N rate of all three cultivars decreased with submergence time and was significantly affected by time (P <0.001) according to the two-way ANOVA test (Table 2). The underwater net photosynthesis was also significantly affected by cultivar (P=0.0010) (Table 2), this was reflected in the initial higher rate for Nerica-4 and the consistency till timepoint 4 for IRRI154. Variation was primarily influenced by time (58.67%) (Table 2).

Table 2: Two-way ANOVA results of underwater net photosynthesis, gas film, porosity, and chlorophyll.

Parameter tested	Source of variation	% of total variation	P value
Underwater net Photosynthesis (P_N)	Interaction	6.57	0.0998
	Time	58.67	<0.0001
	Cultivar	7.20	0.0010
Gas film	Interaction	9.617	0.0007
	Time	65.27	<0.0001
	Cultivar	7.28	<0.0001
Porosity	Interaction	21.09	0.0006
	Time	39.75	<0.0001
	Cultivar	0.12	0.9128
Chlorophyll	Interaction	1.66	0.2669
	Time	87.99	<0.0001
	Cultivar	0.70	0.1230

Difference in loss of gas film between the three cultivars

Gas film was measured in the three rice cultivars during 17 days of submergence. The leaf gas film almost disappeared for all three cultivars during the submergence time (Figure 2). FL478, IRRI154 and Nerica-4 had gas film thickness at respectively 50.63 µm, 42.15 µm and 11.57 µm, at day 0 and 3.23 µm, 1.76 µm and 1.67 µm, at day 17. Time had a significantly effect on thickness of leaf gas film (P <0.0001) (Table 2).
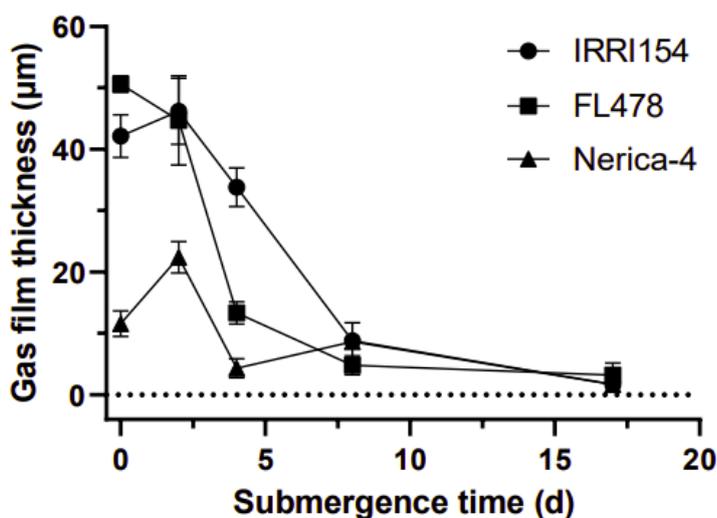

Figure 2: Gas film of leaf segments of *O. glaberimma x O. sativa* 'Nerica-4', *O. sativa* salt tolerant 'FL478' and *O. sativa* salt intolerant 'IRRI154' with time of submergence. Symbols represents means ± SEM, n=4-5.

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The leaf gas films two-way ANOVA test showed significance for time (P<0.0001), cultivar (P <0.0001) and interaction of cultivar and time (P=0.0007) (Table 2). I.e., the leaf gas film was affected differently by the cultivars during the submergence time and the three cultivars did lose their gas film at different pace. Clearly seen in Neric-4's thinner leaf gas film for the first three timepoints (day 0, 2 and 4) compared with the other cultivars. Nerica-4's data point from day 0 was significantly different from IRRI154 (P=0.0028) and FL478 (P=0.0005) (Post-hoc Tukey test). The leaf gas film for the three cultivars lasted a while but were not specifically thick during the last to timepoints of the submergence. Variation was primarily influenced by time (65.27%) (Table 2).

Loss of porosity during submergence time

Porosity was measured on the same leaf segments as gas film. The porosity fell for all three cultivars during the submergence time (Figure 3). IRRI154, Nerica-4 and FL478 had respectively 18.39%, 9.40% and 5.75% porosity at day 0 and 0.49%, 2.31% and 4.74% porosity at day 17. Time had a significant effect on leaf porosity (P <0.0001) (Table 2).
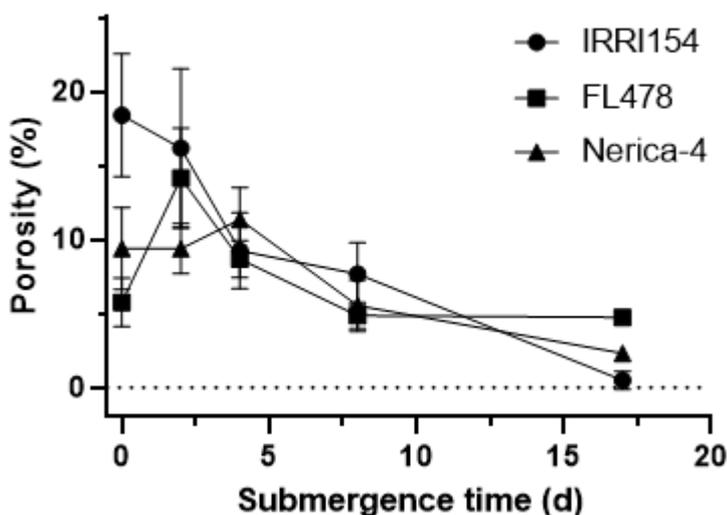

Figure 3: Porosity of leaf segments of *O. glaberimma x O. sativa* 'Nerica-4', *O. sativa* 'FL478' and *O. sativa* 'IRRI154' with time of submergence. Symbols represents means ± SEM, n=4-5.

Compared to Nerica-4 and FL478, IRRI154 had a 95.6% and 219.83% higher porosity (%) before submergence (day 0) (Figure 3). However, around timepoint three (day 4) the three cultivars lost their porosity more similarly (Figure 3). IRRI154 had the lowest porosity at timepoint 5 (day 17) and the post-hoc Tukey test showed significant difference between IRRI154 and respectively FL478 (P<0.0001) and Nerica-4 (P=0.0159). Interaction had a significant effect on leaf porosity (P=0.0006) (Table 2). Variation was primarily influenced by time (39.75%), but also interaction (21.09%) (Table 2).

Decline of chlorophyll concentration similar in all three cultivars

Chlorophyll-a was measured on the same leaf segments as gas film and porosity. The amount of chlorophyll-a in the leaf segments fell for all three cultivars during the submergence time and nearly similar after timepoint three (day 4) (Figure 4). Nerica-4, IRRI154 and FL478 had a chlorophyll concentration at respectively 6.88 µg Chl a/mg DW, 4.74 µg Chl a/mg DW and 4.09 µg Chl a/mg DW at day 0 and 0.03 µg Chl a/mg DW, 0.035 µg Chl a/mg DW and 0 µg Chl a/mg DW at day 17.
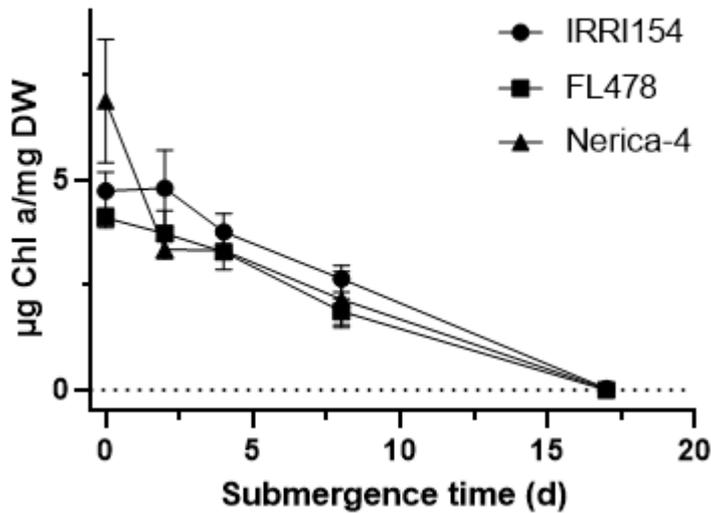

Figure 4: Chlorophyll-a in leaf segments of *O. glaberimma x O. sativa* 'Nerica-4', *O. sativa* 'FL478' and *O. sativa* 'IRRI154' with time of submergence.

Time had a significant effect on chlorophyll-a in the leaf segments ($P < 0.0001$) (Table 2). Variation was primarily influenced by time (87.99%) (Table 2). Nerica-4 had a higher amount of chlorophyll-a before submergence but there were no significant differences in cultivar and amount of chlorophyll ($P = 0.6987$) (Table 2).

The three parameters gas film, porosity and chlorophyll were tested for correlation with underwater net photosynthesis in a nonparametric Spearman correlation. This was done to test if data supports that a decline in underwater P_N was caused by decline in gas film thickness, leaf tissue porosity and/or chlorophyll concentration.

Underwater net photosynthesis and gas film correlation

The data from underwater net photosynthesis was plotted against corresponding gas film thickness and appears to have a coherence (Figure 5). Data points with thicker gas film tended to have a higher underwater net photosynthesis rate than those with a thinner gas film (Figure 5).
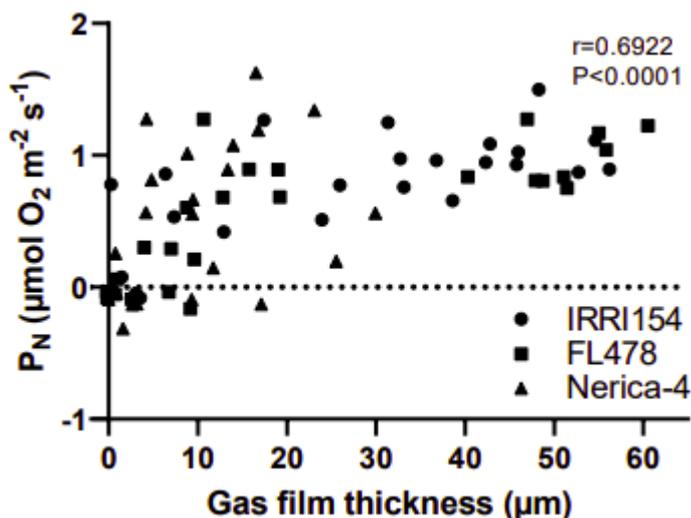

Figure 5: Correlation between underwater net photosynthesis and gas film in leaf segments of *O. glaberimma x O. sativa* 'Nerica-4', *O. sativa* 'FL478' and *O. sativa* 'IRRI154' (r=0.6922, P<0.0001). Some data points (in total 2) are positioned outside the range of the axes. The full dataset was included in the Spearman correlation.

The spearman correlation showed a significant, positive coherence between the thickness of the gas film and the underwater net photosynthesis (Spearman correlation, r = 0.6922 P<0.0001,). The R-value was close to 1, i.e., there was a tendency for the two variables to increase together (GraphPad, 2021). Since the P-value was below 0.05, it is considered probable that the x-variable affects the y-variable. It could also mean that there is another variable that influence both variables (GraphPad, 2021). P_N and gas film both decreased with submergence time (figure 1, figure 2). At timepoint 4 and 5 (day 8 and 17) the gas film had decreased below 9 µm for all three cultivars (figure 2) and at these timepoint the chlorophyll concentration had dropped below 3 µg Chl a/mg DW for the three cultivars (figure 4). The low P_N rates at the same time points as the low gas film thickness could result from low chlorophyll concentration.

Underwater net photosynthesis and porosity

The data from underwater net photosynthesis and porosity appears to have a coherence, since the data points increases for both parameters (Figure 5). The data points with higher porosity (%) tend to have a higher underwater net photosynthesis rate (Figure 6).
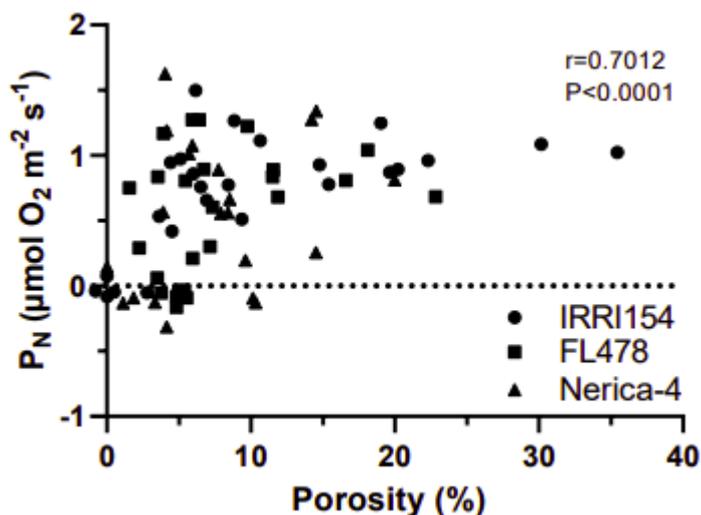

Figure 6: Correlation between underwater net photosynthesis and porosity in leaf segments of *O. glaberimma x O. sativa* 'Nerica-4', *O. sativa* 'FL478' and *O. sativa* 'IRRI154' (r=0.7012), P<0.0001). Some data points (in total 2) are positioned outside the range of the axes. The full dataset was included in the Spearman correlation.

The Spearman correlation showed a significant coherence between the porosity and the underwater net photosynthesis (Spearman correlation, P-value = <0.0001, r = 0.5111).

Underwater net photosynthesis and chlorophyll

The data from underwater net photosynthesis and chlorophyll-a appears to have a coherence (Figure 5). The data points with a low amount of chlorophyll-a tend to have a low underwater net photosynthesis rate and there is an increase of underwater net photosynthesis with increasing amount of chlorophyll-a (Figure 7).
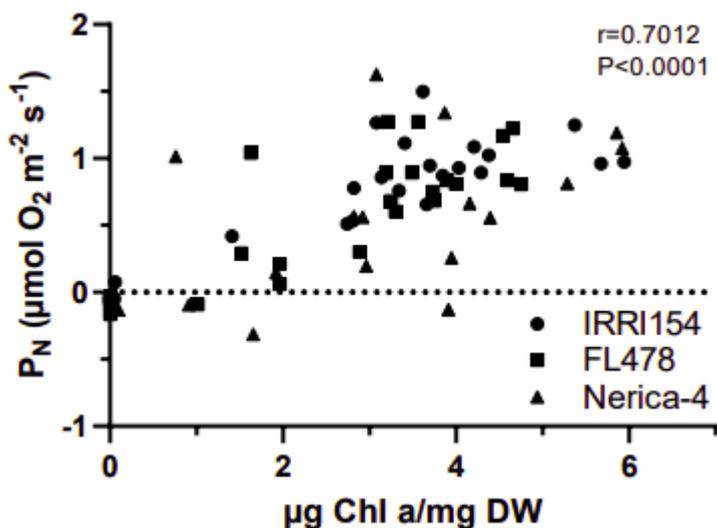

Figure 7: Correlation between underwater net photosynthesis and chlorophyll concentration in leaf segments of *O. glaberimma x O. sativa* 'Nerica-4', *O. sativa* 'FL478' and *O. sativa* 'IRRI154' (r=0.7012, P<0.0001). Some data points (in total 4) are positioned outside the range of the axes. The full dataset was included in the Spearman correlation.

The spearman correlation showed a significant coherence between the amount of chlorophyll and the underwater net photosynthesis (Spearman correlation, P-value = <0.0001, r = 0.7012). The R-value was close to 1 which means there was a tendency for the two variables to increase together (GraphPad, 2021).

Discussion

In this project I assessed the response to complete submergence in the three *Oryza* cultivars Nerica-4, IRRI154 and FL478 by measuring underwater net photosynthesis, leaf gas film thickness, leaf tissue porosity and leaf chlorophyll concentration. The results aligned overall with the initial hypotheses: Leaf gas film did decrease with time of submergence and so did tissue porosity, chlorophyll concentration, and underwater net photosynthesis. This was reflected in the correlation, where gas film, porosity and chlorophyll all were correlated significantly with underwater net photosynthesis. Interestingly Nerica-4 showed a different result by having a thinner gas film compared to IRRI154 and FL478, however it did not differentiate in the other parameters.

In the following paragraphs I will discuss the findings in this project and relate them to previous findings. First, I will discuss the P_N rate in relation to other studies. Thereafter Nerica-4's response to submergence compared to IRRI154 and FL478. Then leaf gas film retention in the three cultivars and its effect on the P_N rate. Last, I will discuss the overall submergence tolerance in the three cultivars in relation to other and further studies.

Low underwater net photosynthesis rates

The results of this study showed surprisingly low P_N rates compared to other studies, especially in the initial measurements. In the study by Winkel *et al*. (2014), measuring underwater net photosynthesis of four rice genotypes, the initial P_N rates were 4-6 μmol O_2 m^{-2} s^{-1} (Winkel *et al.*, 2014) and above 8 μmol O_2 m^{-2} s^{-1} in *Herzog et al.* (2018), where in this project they were 0.96-1.74 μmol O_2 m^{-2} s^{-1}. Possible reasons for this discrepancy could relate to different levels of light and/or temperature during the underwater photosynthesis incubation since this study used the same amount of CO_2 (200 μM CO_2) as the previous studies.

In detail, the light conditions in this project and in the study of Winkel *et al*. (2014) were different as Winkel *et al.,* 2014, incubated leaf segments at 760 μmol photons m^{-2} s^{-1}, whilst this project used 500 μmol photons m^{-2} s^{-1}. P_N increases with the availability of light until a certain level when

photosynthesis becomes light saturated. In rice, light saturation has been described to occur at above 1000 µmol, indicating that an increase in light intensity from 500 µmol photons m^{-2} s^{-1} would increase photosynthesis if no other limitations occur (such as CO_2) (Ye, 2007).

Regarding temperature, leaves were incubated at 30°C in *Winkel et al.* (2014) compared to 25°C in this study. This temperature difference could have an impact on rice underwater P_N, since higher temperature affects the enzyme-activity (Plants in action chap. 14). With higher enzyme-activity there is a higher CO_2 assimilation, enabling a higher P_N rate. In the study by *Colmer & Pedersen* (2007) the temperature is lower, 20°C, than in this project and there is still higher P_N rates, but the enzyme activity and optimum temperatures differs between plants and the effect of temperature on the P_N rate cannot be denied (Plants in action chap. 2014). It should also be noted that temperature in the glass house where the experiments took place reached relatively high day temperatures (occasionally above 40 degrees), so plants might be acclimated to higher temperatures than 25 degrees.

Comparing the obtained underwater photosynthesis rates in rice with other wetland plants, a study by *Colmer & Pedersen* (2007) investigating 6 wetland plants (at 50-500 µM CO_2, PAR 600 µmol photons m^{-2} s^{-1}, and temperature at 20°C) showed P_N rates for three wetland plants with leaf gas film of 0.35-0.80 µmol O_2 m^{-2} s^{-1} at 50 µM CO_2 and 2.50-5.00 µmol O_2 m^{-2} s^{-1} at 500 µM CO_2. While underlining that the available CO_2 is very important for the P_N rate in submerged vegetation, it also indicates that the underwater photosynthesis investigated cultivars would be relatively low in a field situation at low light, even when 200 µM CO_2 is available. This level has been described as an ecological relevant CO_2 concentration in rice field floodwaters (Winkel *et al.,* 2014). The light intensity in the *Colmer & Pedersen* (2007) study does not differ much from this study. In conclusion, it is not straight forward to conclude whether the low underwater P_N rates in the investigated rice cultivars were due to low light, temperature, or a combination.

Thinner leaf gas film in Nerica-4 O. sativa x O. glaberrima than in O. sativa cultivars
Nerica-4 is an *O. sativa* × *O. glaberrima* crossing and was therefore expected to show a different submergence response than the two *O. sativa* cultivars. In fact, Nerica-4 did show a different response to some degree. Nerica-4 had initial higher chlorophyll, which could explain the initial higher P_N. Although Nerica-4 showed initial slightly higher levels of underwater P_N and leaf chlorophyll, for the rest of the time points, Nerica-4 overall followed the same tendency as IRRI154

and FL478. However, Nerica-4 did differ from the two others concerning leaf gas film, where it generally had a thinner gas film, indicating that possibly leaf hydrophobicity in Nerica-4 was lower than in the two *O. sativa* cultivars. The low initial gas film thickness for Nerica-4 does not align with the expectation that thin gas film equals lower P_N rate since it had a higher initial P_N rate than the other cultivars. Even though it had a thinner gas film for the first three timepoints, Nerica-4 did not have a lower P_N rate than the two other cultivars. The thin gas film could be caused by the fact that some of the Nerica-4 plants looked more fatigued at the beginning of the submergence than the two other cultivars. The measured parameters could be affected by plant fitness, so the significantly thinner gas film could be a result of plant fitness and not cultivar differences.

Submergence strategies in O. glaberrima and O. sativa

According to the study by Sakagami, 2012, some *O. glaberrima* varieties are adapted to prolonged submergence and escape response (Sakagami, 2012). This renders plants' sensitivity to short-term flooding, but due to the elongation and therefore improved re-establishment of aerial photosynthesis tolerance toward long-term floods (Sakagami, 2012). I.e., *O. glaberrima* has a higher mortality in flashfloods with rapid increasing water levels, due to depletion of carbohydrates, whilst in slow rising long-term complete submergence having the advantage of shoot elongation (Sakagami, 2012). However, this strategy was not observed in this study, where the *O. sativa* × *O. glaberrima* crossing Nerica-4 did not seem to elongate (personal observation, data not shown), and decrease in P_N rate, tissue porosity or chlorophyll concentration was not faster than the two other cultivars (also not elongating significantly, data not shown).

For further studies it could be interesting to investigate Nerica-4's response to long-term flooding and measure elongation, to see if it responds like *O. glaberrima* in *Sakagami* (2012). This study indicates that Nerica-4 has a just as effective underwater net photosynthesis as two other cultivars despite a thinner gas film, further study of this could be interesting since it differs from other studies about the relation between leaf gas film and under water net photosynthesis (Winkel *et al.,* 2014; Pedersen *et al.,* 2009) and indicates that Nerica-4 might have an effective gas exchange despite the thin gas film.

Leaf gas film

The results showed that the leaf gas film decreased with time of submergence. At time point 4 (day 8) the gas film was thin (4.81-8.67 µm) and at time point 5 (day 17) the leaf gas film was beneath 4 µm for the three cultivars. The gas film persistence showed overall similar patterns as the study by

Winkel et al. (2014*)* (except Nerica-4), where the gas films were diminished by day 7 of submergence in a field situation. However, in the study by *Winkel et al.* (2014) FR13A were the last genotype to lose gas film, which was gone by day 7, where in the results of this project all three cultivars still had gas film by day 8 (4.81-8.67 µm) and a very thin layer by day 17 (1.67-3.23 µm). In the *Winkel et al.* (2014) study, two of the submerged plants were tolerant genotypes FR13A, donor of SUB1 and Swarna-SUB1, introgressed with SUB1 (Winkel *et al.,* 2014). In that study the tolerant genotypes' gas film retention was better than the sensitive genotypes', however the three cultivars in this study had longer gas film retention of approximately 17 days, where the gas films still were positive (1.67-3.23 µm). The initial leaf gas film was thicker in *Winkel et al.* (2014) than in this project. However, in the study by *Winkel et al.* (2014), the rice genotypes were submerged in fields, which could affect the leaf gas film due to more water movement, sediment particles, and lower turbidity in a field compared to a bucket in a glass house, where the rice was placed in this study. Further studies of leaf gas film retention and the processes behind is needed.

Does leaf gas film loss cause senescence of the leaf leading to a decline in underwater net photosynthesis?

The loss of leaf gas film could have led to senescence of the leaf chlorophyll concentration and tissue porosity. The gas film had decreased by timepoint 4 and so had porosity and chlorophyll. Porosity and especially chlorophyll decreased a lot after timepoint 4 where most of the gas film had disappeared, which could indicate that the loss of leaf gas film leads to senescence of the leaves. The P_N rate had decreased a lot by timepoint 4 as well, and it is therefore likely that it is caused by the gas film thickness and senescence of the leaf. The low P_N rates at the same time points as the low gas film thickness could result from low chlorophyll concentration. There was a significant correlation between chlorophyll and underwater net photosynthesis. Another possibility is that they both are affected by another variable, this could be submergence or leaf gas film degradation. The chlorophyll concentration is very similar for the three cultivars, whilst the P_N rate was different. The very different P_N rates for the three cultivars to a similar chlorophyll concentration could indicate that another factor had a bigger impact or that the underwater net photosynthesis is affected by multiple things.

The underwater net photosynthesis had a significant correlation with gas film thickness, tissue porosity and chlorophyll concentration and the positive R-value indicated that underwater net photosynthesis increase with the three factors.

Submergence tolerance of IRRI154, FL478 and Nerica-4

In IRRI154 the porosity, chlorophyll, and gas film datapoints for timepoint 4 was higher than the two other cultivars, this could cause the higher P_N rate for IRRI154 at timepoint 4 compared to Nerica-4 and FL478. IRRI154 held almost the same P_N rate throughout the first 8 days (timepoint 4), where the two other cultivars start to decrease earlier. This indicates that IRRI154 could be more submergence tolerant. Nerica-4 did not show any unexpected response, except its high P_N rate at low gas film thickness, that could be caused by the high amount of chlorophyll at the same timepoint (day 0).

In conclusion, compared to the SUB1 genotypes FR13A and Swarna-SUB1 from the study *Winkel el al.* (2017), the cultivars of this study did not seem markedly more submergence tolerant, based on P_N rates, chlorophyll concentration and gas film thickness. However, leaf gas films did last a bit longer for the cultivars used in this study.

Conclusion and outlook

The project showed that the three cultivars IRRI154, FL478 and Nerica-4 all experienced decrease in underwater net photosynthesis, gas film thickness, tissue porosity and chlorophyll concentration with submergence time. Nerica-4 differed from the other cultivars with a thinner gas film, but overall followed the same tendency as IRRI154 and FL478. The cultivars had low underwater net photosynthesis rates, probably due to conditions during incubation, and compared with other *O. sativa* genotypes not markedly more submergence tolerant. However, gas film persisted 6-10 days longer in this study than other studies assessing leaf gas film persistence, which could warrant further research into the reasons for the longer leaf gas film persistence observed in this study. The three parameters gas film thickness, tissue porosity and chlorophyll concentration all significantly correlated with underwater net photosynthesis, underlining the importance in delaying leaf senescence in order to improve rice submergence tolerance.

The four parameters measured in this project only partially describe rice submergence tolerance. To get a full picture of the submergence tolerance of the three cultivars: IRRI154, FL478 and Nerica-4, effects of submergence on plant survival (following a post-submergence recovery period), growth and traits such as internal aeration and the radial O_2 loss from roots could be examined as they play an important role for plant submergence tolerance (Colmer & Voesnek 2009; Colmer 2003). In

addition, measurements of shoot elongation during submergence could allow categorization of cultivar submergence strategies into LOES or LOQS response-types.

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