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Original Article

Leaf gas films contribute to rice (*Oryza sativa*) submergence tolerance during saline floods

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ABSTRACT

Floods and salinization of agricultural land adversely impact global rice production. We investigated whether gas films on leaves of submerged rice delay salt entry during saline submergence. Two-week-old plants with leaf gas films (+GF) or with gas films experimentally removed (−GF) were submerged in artificial floodwater with 0 or 50 mM NaCl for up to 16 d. Gas films were present >9 d on GF plants after which gas films were diminished. Tissue ion analysis (Na^+, Cl^- and K^+) showed that gas films caused some delay of Na^+ entry, as leaf Na^+ concentration was 36–42% higher in −GF leaves than +GF leaves on days 1–5. However, significant net uptakes of Na^+ and Cl^-, and K^+ net loss, occurred despite the presence of gas films, indicating the likely presence of some leaf-to-floodwater contact, so that the gas layer must not have completely separated the leaf surfaces from the water. Natural loss and removal of gas films resulted in severe declines in growth, underwater photosynthesis, chlorophyll$_a$ and tissue porosity. Submergence was more detrimental to leaf P_N and growth than the additional effect of 50 mM NaCl, as salt did not significantly affect underwater P_N at 200 μM CO_2 nor growth.

Key-words: flooding; leaf Cl^-; leaf K^+; leaf Na^+; plant submergence tolerance; salinity; salt intrusion.

INTRODUCTION

Floods annually affect large areas of farmlands worldwide and cause severe crop losses when plants become submerged (Jackson 2004). Crop damage is mainly caused by the hampered gas exchange between plants and floodwater because of a 10^4-fold slower gas diffusion and low solubility of O_2 in water compared with that in air (Armstrong 1979; Voesenek *et al.* 2006). Paddy field rice is adapted to growth in anoxic soils and therefore is tolerant to soil waterlogging and even partial shoot submergence (Colmer *et al.* 2014; Kirk *et al.* 2014). However, only a few days of complete submergence can lead to severe damage and death of rice (Das *et al.* 2009), but with important differences among rice genotypes (Ismail *et al.* 2013). The restricted gas exchange impedes respiration and photosynthesis (also because of low light) in submerged shoots (Mommer & Visser 2005) while the consumption of soluble carbohydrates (Setter *et al.* 1997) further depletes tissue sugars and energy (if shoots elongate). The factors described previously contribute to damage during floods, together with at desubmergence water deficits (Setter *et al.* 2010) and oxidative stress (Bailey-Serres & Voesenek 2008) that can result in further damage and even death.

Floodwaters may contain NaCl, and salinity is a major impediment to increasing global rice production (Negrão *et al.* 2011) as rice is a salt-sensitive species. For rice with shoots in air, salinity above 30 mM NaCl results in yield decreases by 12% for each ~10 mM NaCl increase (Grieve *et al.* 2012). Salinity imposes both an osmotic stress on the plant because of high solute concentrations outside cells, as well as ion-specific stresses caused by high Na^+ and Cl^- concentrations in plant tissues (Munns & Tester 2008; Negrão *et al.* 2011). The need to improve rice salinity and submergence tolerance is further urged by climate change causing rising seawater levels and lower river flows, leading to seawater inundation of large rice growing regions such as the Vietnamese Mekong Delta (Wassmann *et al.* 2004). A second example is that the salinity affected areas in Bangladesh increased from about 83 million ha in 1973 to 106 million ha in 2009 (Sinha *et al.* 2014). Thus, efforts are being made to combine submergence tolerance and salinity tolerance in the so-called climate-smart rice (De Ocampo *et al.* 2013; IRRI 2016).

Rice leaves are surrounded by a gas film (initial average thickness of 50–62 μm, Pedersen *et al.* 2009; Winkel *et al.* 2013; Winkel *et al.* 2014) for up to 6 d during submergence in the field (Winkel *et al.* 2014). Presence of such gas films delayed salt entry into submerged leaves of *Melilotus siculus* (Teakle *et al.* 2014), but this is the only study to have evaluated this effect. Leaf gas films have been shown to enhance underwater photosynthesis of rice, dark respiration, root pO_2 and growth, by greatly enhancing gas exchange between leaves and floodwater (Pedersen *et al.* 2009; Winkel *et al.* 2013; Verboven *et al.* 2014; Winkel *et al.* 2014), thereby contributing to rice submergence tolerance. Our main objective was to test the effect of the presence of leaf gas films on salt entry into submerged

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rice; we hypothesized that Na^+ and Cl^- entry, and K^+ loss, would be delayed by the presence of leaf gas films acting as an 'insulating' physical barrier between each leaf and saline floodwater.

MATERIALS AND METHODS

Plant culture

Seeds of rice (*Oryza sativa* L. var. Amaroo) were germinated following Mongon *et al.* (2014). Dehulled seeds (i.e. caryopses) were washed with dilute sodium hypochlorite (0.1%) for 30 s, rinsed in deionized (DI) water and then imbibed in aerated 0.5 mM $CaSO_4$ for 3 h. The seeds were placed on a plastic mesh floating on a 10% strength nutrient solution (for chemical composition, see below) in darkness. After 4 d, the seedlings were transferred to a 25% strength nutrient solution and exposed to light. Seven days after imbibition, the seedlings were transplanted to 4 L plastic pots with perforated lids (eight plants per pot) containing 100% strength nutrient solution. Plants were held individually in each of the eight holes in the lids using polyethylene foam, and the pots were covered with aluminium foil to exclude light from the root system. Eleven days after imbibition (2–3 d before submergence), roots received a hypoxic pretreatment by flushing the nutrient solution with N_2 gas for 5 min. On the day before submergence, plants were transferred to 2.2 L pots (four plants per pot) containing 100% strength nutrient solution, with additional 2.5 mM NH_4NO_3, made stagnant with 0.1% (w/v) agar and previously deoxygenated by flushing overnight with N_2 gas.

The composition of the nutrient solution at 100% strength was as follows: KNO_3, 3.75 mM; NH_4NO_3, 0.625 mM (plus 2.5 mM NH_4NO_3 when stagnant agar was used); KH_2PO_4, 0.2 mM; $MgSO_4.2H_2O$, 0.40 mM; $Na_2O_3Si.9H_2O$, 0.10 mM; $CaSO_4.2H_2O$, 1.5 mM; KCl, 100 μM; H_3BO_3, 50 μM; $MnSO_4.H_2O$, 4.0 μM; $ZnSO_4.7H_2O$, 4.0 μM; $CuSO_4.5H_2O$, 1.0 μM; $Na_2MoO_4.2H_2O$, 1.0 μM; $NiSO_4.7H_2O$, 2.0 μM; and Fe-EDTA, 50 μM. The solution also contained 2.5 mM MES buffer, and the pH was adjusted to 6.5 using KOH. At 7–8 d after imbibition, one dose of $FeSO_4.7H_2O$ was added to each 4 L pot to a final concentration of 5.0 μM to avoid any iron deficiency in the seedlings. The nutrient solution in the pots was replaced with fresh solution every 6 d during the entire experiment and topped up with DI water as required to replace water consumed in transpiration. Plants were kept in a naturally lit, temperature-controlled (30/25 °C day/night) phytotron during October to November 2015 in Perth, Western Australia. Light in the phytotron was 741 μmol photons $m^{-2}s^{-1}$ at midday even on a cloudy day.

Experiment 1 – tissue ions in +GF and −GF plants during 16 d submergence in non-saline and saline (50 mM NaCl) artificial floodwaters

Plants were grown in two batches staggered with time, owing to the limited number of cylinders in the submergence systems (described next). Submergence treatments commenced 13–15 d after imbibition when all plants had a visible fourth leaf collar. The setup of the submergence system has been described previously (Pedersen *et al.* 2009; Teakle *et al.* 2014). In short, the 2.2 L pots each containing four plants were randomly transferred to 12 L clear Perspex cylinders filled with either saline (50 mM NaCl) or non-saline (0 mM NaCl) submergence solution. The basal submergence solution (artificial floodwater) contained the following: $CaSO_4$, 2.0 mM; $MgSO_4$, 0.25 mM; and $KHCO_3$, 2.0 mM. The root medium in all cases was non-saline.

Cylinders filled with saline or non-saline submergence solution were connected to two separate, identical lines of aquarium pumps and ultraviolet (UV)-filters (JBL AquaCristal UV-C; JBL GmbH & Co. KG, Neuhofen, Germany). In each system of nine Perspex cylinders per line, a pH controller (JBL CO_2/pH Control; JBL GmbH & Co. KG, Neuhofen, Germany) connected to a cylinder with pressurized CO_2 maintained free CO_2 at 200 μM by referring to the relevant pH set points for non-saline (pH 7.3, Mackereth *et al.* 1979) and saline (pH 7.1, Pierrot *et al.* 2006) water. Dark plastic covered the lids of the pots and the bottom and basal sides of the cylinders, excluding light from entering basal portion of each cylinder that contained the plastic pots with the nutrient solution. Rubber-covered weights weighed down the pot in each cylinder. Plants grown in identical pots with nutrient solution and rubber weights were placed in empty cylinders (i.e. containing air) and with plastic mesh near the top of each cylinder (see below for the reason this mesh was needed especially for the submerged plants), serving as 'emergent' controls with shoots in air. Light in the water-filled cylinders was 863 μmol photons $m^{-2}s^{-1}$ at midday on a cloudy day, which was above values in air (see section 'Plant culture'); this could be caused by filled cylinders acting as a lens thereby focusing light onto the light sensor (Walz US-SQS/L; Heinz Walz GmbH, Effeltrich, Germany).

Before submergence, plants were either untreated, thus retaining clearly visible leaf gas films upon submergence (+GF), or the entire shoot was brushed with 0.1% (v/v) Triton X-100 (Colmer & Pedersen 2008; Pedersen *et al.* 2009; Winkel *et al.* 2013) preventing leaf gas film formation when submerged (−GF). Shoots treated with 0.1% Triton X-100 were rinsed using a separate batch of submergence solution prior to insertion of these plants into the cylinders. New leaves formed during the submergence period were brushed with 0.1% Triton X-100 and rinsed with a separate batch of submergence solution, when plants were raised out of the tanks for this process every 2 d. Plastic mesh held 20 mm below the water surface within each cylinder prevented leaf emergence into the air above the water when shoots elongated following submergence. The submergence treatment lasted 9 d for plants treated with 0.1% Triton X-100 (without leaf gas films) and 16 d for plants initially retaining leaf gas films. The shorter treatment period of the 0.1% Triton X-100 treated plants was due to the beginning of some disintegration of the leaves after 9 d of submergence (observed during a pilot experiment). Plants were harvested on days 0, 1, 2, 5, 9 and 16 of the submergence treatment. The youngest fully expanded leaves at time of submergence (leaf 4) and leaf 3 were excised and used for further analysis (the entire third leaf blade was used for tissue ion analysis;

the fourth leaf was used for measuring underwater photosynthesis, leaf gas film thickness, leaf tissue porosity, scanning electron microscopy, chlorophyll concentration and tissue ion analysis). Details of measurements are given next.

Growth

Plants were harvested for dry mass (DM) measurements on days 0, 9 and 16 of submergence treatments. Plants were separated into shoot and roots and oven dried at 60 °C for 48 h before weighing. As the experiment consisted of two different batches of plants, we calculated relative growth rates $RGR = (\ln W_2 - \ln W_1)/(t_1 - t_0)$ for growth comparisons, where W_1 and W_2 are the initial and final weight (g), respectively, and t_1 and t_2 are the initial and final time (days), respectively.

Recovery was assessed following desubmergence after 9 d of submergence. Four pots each containing four plants were desubmerged and placed in empty Perspex cylinders. After 10 d with shoots again in air, the plants were scored for survival, dead and living shoot tissues were separated, samples were dried at 60 °C for 48 h, and DM was recorded.

Underwater net photosynthesis

Underwater net photosynthesis (P_N) was measured following the approach described in Pedersen *et al.* (2013). Leaf segments were incubated in a defined medium (described next) for a known time in closed transparent glass vials with gentle mixing and held at a constant temperature in light [photosynthetically active radiation (PAR) given next], after which the O_2 evolution (P_N) by the leaf segments was measured against a blank vial lacking leaf segments. Four replicate leaves (youngest fully expanded at the time of submergence from four different plants) were taken from each of the two treatments (non-saline or saline submergence). Leaf segments of 10 mm in length (projected area ~50 mm^2) were excised from the top third of the lamina. Underwater P_N was measured at 30 °C using 25 mL glass vials with two glass beads added to provide mixing as the vials were held on a 'turning wheel' during incubation with PAR inside the vials of 1000 μmol photons $m^{-2} s^{-1}$ provided from a vertically positioned light-emitting diode lamp (Valoya R300 NS1; Valoya Ltd., Helsinki, Finland) providing 94% of PAR with a colour temperature of 4800°K. Measurements were performed during the same time of day (1000–1400 h) on all days.

Following incubations of known duration (90–120 min), dissolved O_2 concentration in each vial was measured using an O_2 optode (Unisense OP-MR; Unisense A/S, Aarhus, Denmark) connected to an optode meter (Unisense Micro-Optode meter). The optode was calibrated at 30.0 °C in water at air equilibrium (20.6 kPa O_2) and in anoxic water (0.0 kPa O_2) containing 100 mM sodium ascorbate and 100 mM NaOH. Projected area of each individual leaf segment was measured using digital photos and analysis in ImageJ (Schneider *et al.* 2012). Samples were then immediately frozen at −20 °C, freeze-dried and DM recorded.

Leaf gas film thickness and tissue porosity

The leaf gas film volume and tissue gas-filled porosity were measured using the 'buoyancy method' (Raskin 1983; Thomson *et al.* 1990) on 50 mm segments of the fourth leaf according to Winkel *et al.* (2013) at room temperature. The leaf segment area was measured as described for 'Underwater net photosynthesis', frozen at −20 °C, freeze-dried and DM recorded. Mean gas film thickness was calculated by dividing gas film volume (mm^3) with the two-sided area (mm^2).

Tissue ion concentrations

In order to retrieve sufficient tissue for ion concentration analysis, the entire third leaf and the remaining ~30–80 mm of the fourth leaf were excised from submerged plants and rinsed for 5–10 s in DI water. Leaf Na^+, Cl^- and K^+ concentrations were determined following Munns *et al.* (2010). In short, oven-dried (60 °C) leaf samples were extracted in 2.5–5 mL 0.5 mM HNO_3 for 2 d at 25 °C. Extracts were diluted with Milli-Q water as required and analysed for Na^+ and K^+ (Jenway PFP7 Flame Photometer, Jenway, Essex, UK) and Cl^- (Slamed Chloridometer CHL 50, Slamed ING GmbH, Frankfurt, Germany). The reliability of these analyses was confirmed by taking a reference plant sample (ASPAC no. 85) with known ionic composition through the same procedures.

Chlorophyll concentration

The freeze-dried leaf segments (from underwater P_N measurements) were each homogenized in a 2 mL Eppendorf tube using two metal beads for 10 s on a mini bead-beater (Mini Bead Beater; BioSpec Products Inc., Bartlesville, OK, USA). Chlorophyll was extracted for 24 h in 96% ethanol, centrifuged at 9000 rpm for 3 min and $chlorophyll_a$ absorbance measured at 656 and 750 nm on a spectrophotometer (Shimadzu UV-1800; Shimadzu Corp., Kyoto, Japan). $Chlorophyll_a$ concentrations were calculated using equations of Mackinney (1941).

Scanning electron microscopy

Leaf segments were frozen immediately after sampling and then freeze-dried. Samples were gold-coated in a sputter coater for 90 s and then analysed using a scanning electron microscope (FEI Inspect S; FEI Company, Hillsboro, OR, USA) at high vacuum mode, 12.5 kV and 500–7000 × magnification. For closer examination of wax platelets, samples were also analysed with a field emission scanning electron microscope (JEOL JSM-6335F, JEOL Ltd., Peabody, MA, USA) at 7.0 kV and 27–45.000 × magnification.

Experiment 2 – the effect of pO_2 on leaf ion concentrations

To evaluate the effect of O_2 supply to submerged leaves on tissue ion net uptake or loss, excised leaves were subject to 24 h incubation in darkness in saline water with pO_2 set to five different levels (described next). Plants were grown to the same

age and developmental stage as in experiment 1 (15-day-old-plants). The youngest fully expanded leaf was excised and either treated with 0.1% (v/v) Triton X-100 in non-saline submergence solution (and rinsed with non-saline submergence solution, −GF) or left untreated as controls (+GF). The submergence solution composition was as described for experiment 1. The cut end was sealed using Vaseline. One treated (−GF) and one control (+GF) leaf were placed pairwise in four 250 mL conical flasks for each pO_2 treatment containing saline submergence solution (basal submergence solution plus 50 mM NaCl) as described previously for experiment 1; each leaf was weighed-down under the solution by a plastic-coated paper clip. Two flow controllers (Bronkhorst High-Tech B.V. series with B.V. E-5700 power supply; Bronkhorst High-Tech B.V., Ruurlo, the Netherlands) connected to a pressurized N_2 cylinder and an air pump were used to adjust pO_2 in the submergence solution to 0.01, 0.46, 1.59, 3.16 and 20.23 kPa O_2. Leaves were incubated in the dark for 24 h at 25 °C. After incubation, leaves were visually inspected for presence/absence of leaf gas films and then rinsed and analysed for tissue ion concentrations as described previously. Ion uptake rates were calculated using initial tissue ion concentrations from the same leaf type sampled from plants in experiment 1; these initial concentrations were then subtracted from the final concentrations and divided by the incubation time (24 h).

Data analysis

Data were analysed with GRAPHPAD PRISM version 6.07 (GraphPad Software, La Jolla, CA, USA), SYSTAT version 12.02 (Systat Software Inc., San Jose, CA, USA) and SPSS version 22 (SPSS Inc., Chicago, IL, USA) for Windows statistical software. Normally distributed data were analysed using two-way or three-way ANOVA; data requiring transformations are specified next. Variance homogeneity was confirmed by visual inspections of residual plots and Levene's test for variance homogeneity ($P > 0.05$). Correlations (Fig. 4 and Supporting Information Figs S4 and S5) were analysed by calculating non-parametric Spearman rank correlation coefficients because of lack of bivariate data normality and relationships being non-linear. Significance level of $P < 0.05$ was used for all analyses. For ANOVA analyses, a *post hoc* Sidak or Tukey test was performed when significant effects were found.

Leaf gas film thickness, chlorophyll$_a$ and leaf porosity data (Fig. 1) were analysed using two-way ANOVA with 'time' and 'salt' as fixed factors (days 1–16 of treatments). Measurements performed on day 0 (initials) were excluded, and leaf gas film thickness and leaf porosity data were log and square-root transformed, respectively, in order to improve variance homogeneity. Leaf porosity variances were, however, still significantly different (Levene's test, $P = 0.033$), but as sample sizes were equal making ANOVA robust to unequal variances (Prophet Statguide 1997; Graphpad Software Inc. 2013) and after visual inspection of residual plots, we considered application of ANOVA on transformed data appropriate.

Tissue ion concentrations from experiment 1 (Fig. 2) were analysed using two-way ANOVA with 'time' and 'gas film' as

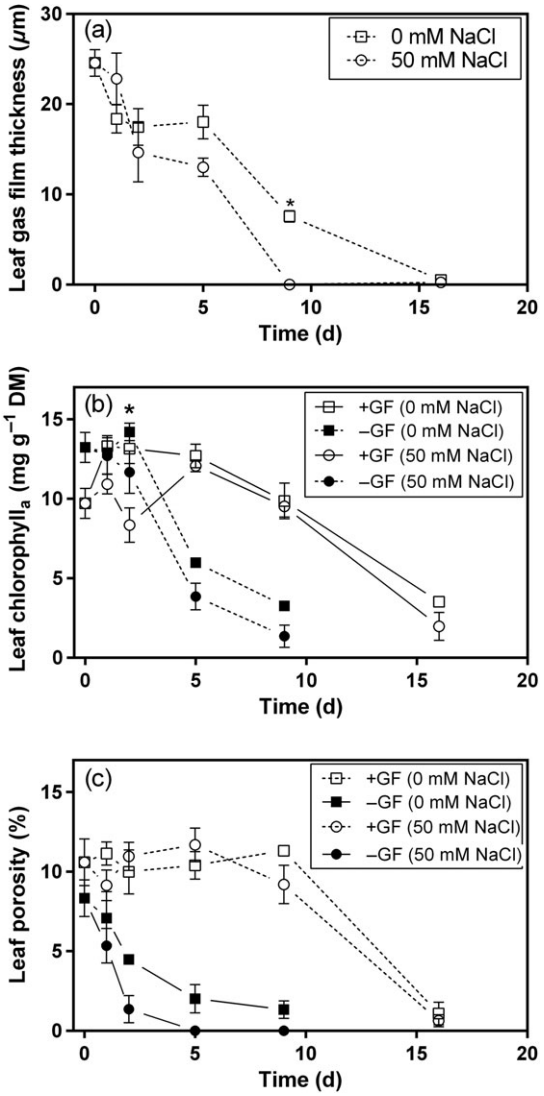

Figure 1. Gas film thickness (a), chlorophyll$_a$ (b) and tissue porosity (c) of the youngest fully expanded leaf with time of submergence of rice in water (containing basal ions, see Methods) with 0 mM NaCl (squares) or 50 mM NaCl (circles) for plants with leaf gas films (+GF, open symbols) or treated with 0.1% Triton X-100 and without gas films (−GF, closed symbols). The +GF and −GF plants are from different batches; hence, these have separate initials. Roots were in non-saline nutrient solution. In (a), two-way ANOVA on log-transformed data (days 1–16) showed a significant time × salt interaction ($P < 0.0001$). * denotes significant difference (Sidak's multiple comparisons test, $P < 0.05$). Gas film thickness on plants that had been brushed with 0.1% Triton X-100 remained between 0.0 and 0.4 μm throughout the experiment, and gas film thickness on initials and emergent controls (i.e. in both cases, leaves from shoots in air were submerged and immediately measured) at end of treatment (day 16) did not differ significantly (*t*-test, $P = 0.332$; data not shown). In (b), two-way time × salt ANOVA (days 1–16) showed significant salt and time effects in both +GF and −GF treatments ($P < 0.01$). * denotes significant difference at 0 and 50 mM NaCl, within each GF treatment (Sidak's multiple comparisons test, $P < 0.05$). In (c), two-way time × salt ANOVA on square root-transformed data (days 1–16) showed significant time effect for +GF ($P = 0.0001$) and significant time ($P < 0.0001$) and salt ($P = 0.0017$) effect for −GF.* denotes significant difference between 0 and 50 mM NaCl within GF treatments (Sidak's multiple comparisons test, $P < 0.05$). Values are means (±SE, $n = 3$–4).

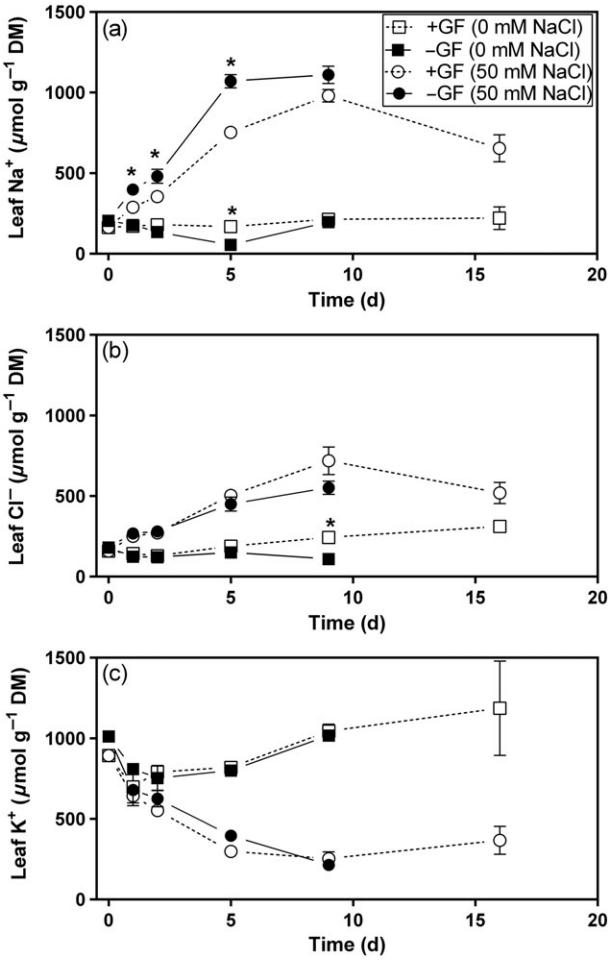

Figure 2. Leaf Na^+ (a), Cl^- (b) and K^+ (c) concentrations with time of submergence of rice in water (containing basal ions, see Methods) with 0 mM NaCl (squares) or 50 mM NaCl (circles) for plants with leaf gas films (+GF, open symbols) or treated with 0.1% Triton X-100 and without gas films (−GF, closed symbols). Samples were the entire third and part of the fourth leaf (see Methods). Roots were in non-saline nutrient solution. * denotes significant difference between means of +GF and −GF within an NaCl treatment (Sidak's multiple comparisons test, $P < 0.05$). The GF had no significant effect on leaf K^+ or log-transformed Cl^- concentrations during submergence in 50 mM NaCl using two-way time × GF ANOVA (days 1–9, $P > 0.05$), but time did ($P < 0.0001$). Two-way ANOVA on log-transformed Na^+ concentrations (days 1–9) showed significant GF ($P < 0.0001$) and time ($P < 0.0001$) effects. The +GF and −GF plants are from different batches; hence, these have separate initials. On day 16, ion concentrations in emergent controls in air (roots in non-saline nutrient solution) did not differ significantly from initials (leaf tissue ion concentrations of emergent controls were 180, 137 and 792 μmol Na^+, Cl^- and K^+ g^{-1} DM, respectively, $P > 0.05$, Tukey's multiple comparisons test, data not shown). Values are means (±SE, $n = 3$–4 except Na^+ on day 5 at 0 mM NaCl (−GF) where $n = 1$ because of a sampling error).

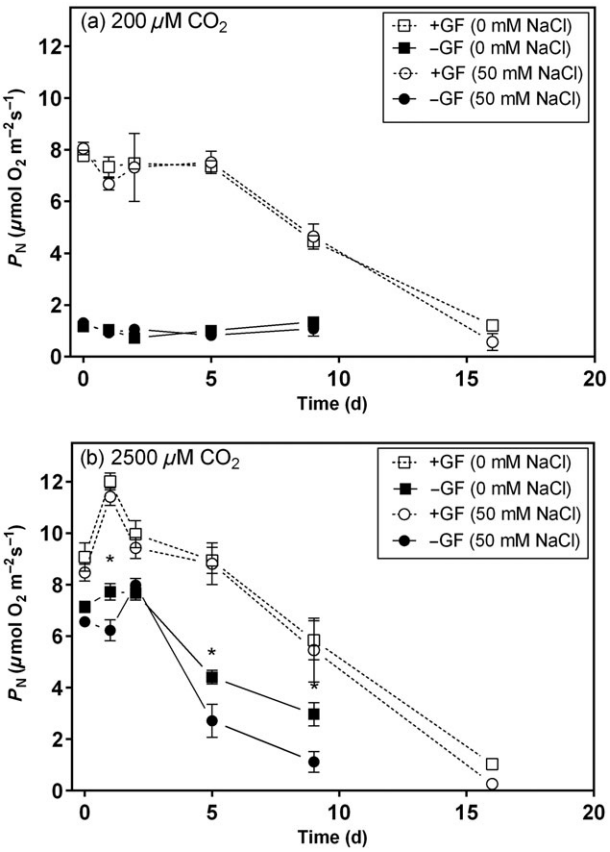

Figure 3. Underwater net photosynthesis (P_N, youngest fully expanded leaf) with time of submergence of rice in water (containing basal ions, see Methods) with 200 (a) or 2500 (b) μM CO_2 and 0 (squares) or 50 (circles) mM NaCl for plants with leaf gas films (+GF, open symbols) or treated with 0.1% Triton X-100 and without gas films (−GF, closed symbols). Roots were in non-saline nutrient solution. Three-way time × salt × GF ANOVA performed for 200 and 2500 μM CO_2, respectively, showed significant salt effect only at 2500 μM CO_2 ($P = 0.002$). There was a significant time × GF interaction at both 200 and 2500 μM CO_2 ($P < 0.01$).* denotes significant difference between 0 and 50 mM NaCl within the same CO_2 and GF treatment (Sidak's multiple comparisons test, $P < 0.05$). Values are means (±SE, $n = 3$–4).

fixed factors (days 1–9 of treatments). Initials were excluded, and Na^+ and Cl^- concentrations were log-transformed in order to improve variance homogeneity. Underwater P_N (Fig. 3) was analysed separately for 2500 and 200 μM free CO_2, resulting in a three-way ANOVA with 'time', 'salt' and 'gas film' as fixed factors. RGR (Fig. 5) and amount of dead shoot DM (Fig. 6) were analysed using two-way ANOVA with 'salt' and 'gas film' as fixed factors. Tissue ions from experiment 2 (Fig. 7 and Supporting Information Fig. S6) were analysed using two-way ANOVA with 'gas film' and 'pO_2' as fixed factors. Shoot length and tiller number (Supporting Information Table S1) were analysed using one-way ANOVA and non-parametric Kruskal–Wallis test, respectively, as transformation of tiller number was unable to ensure variance homogeneity.

RESULTS

To investigate the effects of gas films on leaves of rice submerged in saline water, we compared tissue ion concentrations and other parameters for plants retaining leaf gas films or where the gas films had been experimentally removed.

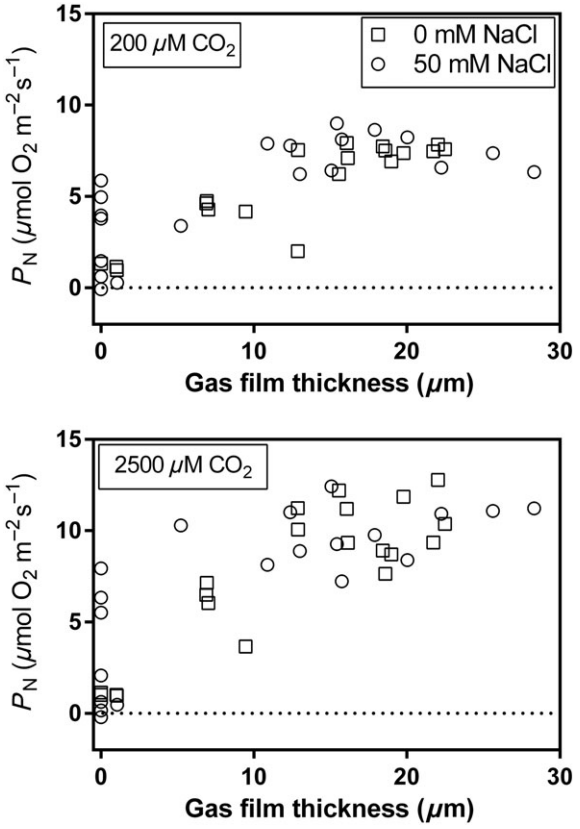

Figure 4. Underwater photosynthesis (P_N, youngest fully expanded leaf) at 200 and 2500 μM CO_2 of rice leaves retaining leaf gas films submerged for 1–16 d in water (containing basal ions, see Methods) with 0 (circles) or 50 (squares) mM NaCl, plotted against the corresponding leaf gas film thickness. Roots were in non-saline nutrient solution. *r* values from non-parametric Spearman rank correlation analysis, * denoting levels of significance (levels of $P > 0.05$, $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$ or $P \leq 0.0001$ are denoted by n.s., *, **, ***, ****, respectively): 200 μM CO_2, $r = 0.7279$****; 2500 μM CO_2, $r = 0.7476$****. Values are means as presented in Figs 1 and 3.

Experiment 1 had non-saline and saline (50 mM NaCl, ~5 dS m^{-1}) artificial floodwater, a level that allowed salinity to be imposed in one step without an 'osmotic shock' and 50 mM NaCl resulted in substantial Na^+ entry into the shoot of rice at the early seedling stage (var. Amaroo; Kurniasih *et al.* 2013); this same variety was used in the present study. Experiment 2 investigated whether O_2 status affects ion net uptake or loss by incubating excised leaves of rice in saline (50 mM NaCl) artificial floodwater at a range of pO_2. In the first section followed, we describe the retention time of gas films and changes in leaf tissue Na^+, Cl^- and K^+ in plants with intact gas films (+GF) compared with plants with gas films removed by brushing with dilute Triton X-100 (−GF) immediately prior to submergence in artificial floodwater with 0 and 50 mM NaCl (experiment 1). We then report on leaf $chlorophyll_a$, leaf porosity, underwater P_N and growth of submerged plants (experiment 1). Finally, we describe the effects of varying pO_2 on net uptake or loss of ions by submerged leaves with or without gas films (experiment 2).

Gas films: retention duration and influence on leaf tissue ions during saline submergence

Rice leaves retained a clearly visible gas film when submerged. Initially, mean gas film thickness was 25 μm (Fig. 1). Gas film thickness declined to 18 μm (0 mM NaCl) and 13 μm (50 mM NaCl) during the first 5 d of submergence, followed by earlier loss of gas films in saline water (after day 5 and before day 9) than in non-saline water (after day 9 and before day 16). It should be noted that other studies report initial rice leaf gas film thickness ranging from 50 to 62 μm in five genotypes (Pedersen *et al.* 2009; Winkel *et al.* 2013; Winkel *et al.* 2014), that is, twice as thick as in the present study (Fig. 1). As one of these studies was performed on var. Amaroo (of similar age as in present study), this difference should not necessarily be interpreted as sign of genotypic variation but could result from environmental conditions (e.g. temperature during measurements, which differed with 10 °C) or different leaf sections used.

Adaxial sides of leaves showed similar macro-structures, micro-structures and nano-structures considered responsible for leaf hydrophobicity (grooves, papillae and wax platelets, respectively) prior to and after loss of gas films (Supporting Information Figs S1 and S2), with the exception of wax platelets located on papillae that showed some slight changes. These wax platelets appeared to be more rounded after loss of leaf hydrophobicity at 50 mM NaCl compared with the initials (Supporting Information Fig. S2b,c). From day 9, leaves were increasingly covered by filaments, possibly from filamentous epiphytic algae (Supporting Information Fig. S1).

To evaluate the effect of gas film removal on tissue ions, we measured Na^+, Cl^- and K^+ in the third leaf and part of the fourth leaf sampled from various plants at 4–5 time points during 9–16 d of submergence. Na^+ uptake was substantial from day 1 even in leaves with a gas film, with tissue Na^+ concentration having increased 4.6-fold (+GF and gas films still present) on day 5 relative to initials. Leaf Na^+ increased even more (36–42% greater in −GF than +GF plants on days 1, 2 and 5; Fig. 2) when plants had their gas films removed prior to submergence in saline water. Consequently, two-way ANOVA showed significant gas film (and time) effects for Na^+ (see caption of Fig. 2). Tissue Na^+ increased to 1111 μmol Na^+ g^{-1} DM in −GF plants on day 9, but on this single time point, Na^+ was not significantly higher than the 981 μmol Na^+ g^{-1} DM measured in plants initially possessing a gas film (note that gas films were no longer present at this sampling time). When tissue Na^+ is expressed as a concentration in tissue water (Supporting Information Fig. S3) rather than on a DM basis, Na^+ accumulated to 175 mM in −GF plants and 172 mM in +GF plants on day 5 at 50 mM NaCl (not significantly different).

Surprisingly, experimental removal of gas films did not significantly affect leaf tissue Cl^- or K^+ concentrations of submerged plants at 50 mM NaCl (Fig. 2). On day 5 when gas films were still present on the +GF plants, both +GF and −GF leaves only retained 33% of initial K^+ concentrations. K^+ loss resulted in minimum tissue K^+ of 215 μmol K^+ g^{-1} DM (27 mM in tissue water, −GF, day 9; Supporting Information Fig. S3). Cl^- concentrations had increased threefold in

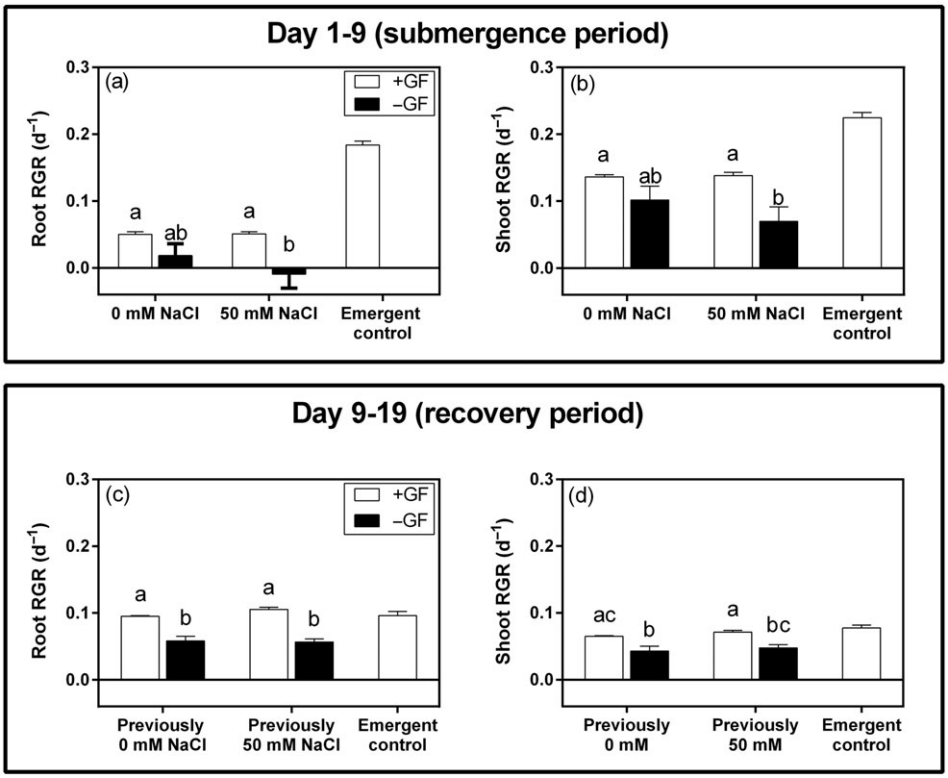

Figure 5. Relative growth rates (RGR) during 9 d submergence (top panel) and the following 10 d recovery period (bottom panel) in roots (a, c) and shoots (b, d) of rice plants retaining leaf gas films (+GF, open bars) or treated with 0.1% Triton X-100 and without leaf gas films (−GF, closed bars) in water (containing basal ions, see Methods) with 0 or 50 mM NaCl. Roots were in non-saline nutrient solution. Letters denote significant differences between columns (Tukey's multiple comparisons test, $P < 0.05$). In all four datasets, two-way salt × GF ANOVA only detected significant effects of gas film ($P < 0.01$) but not salt ($P \geq 0.2598$). The +GF and −GF were batches staggered with time, and the emergent control columns are therefore the mean RGR of these two columns. Values are means (±SE, $n = 4$).

both +GF and −GF leaves on day 5, with maximum tissue Cl^- of 719 μmol Cl^- g^{-1} DM on day 9 (+GF, 124 mM in tissue water; Supporting Information Fig. S3). Consequently, two-way ANOVA on leaf Cl^- and K^+ concentrations only showed significant time effects, contrasting to the additional gas film effect found for leaf Na^+ concentrations (see caption of Fig. 2).

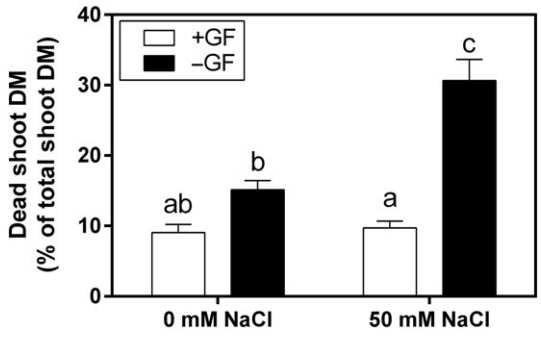

Figure 6. Dead shoot tissue as percent of total shoot dry mass of rice after submergence in water (containing basal ions, see Methods) with 0 or 50 mM NaCl and a following 10 d recovery period of plants with leaf gas films (+GF, open bars) or treated with 0.1% Triton X-100 and without gas films (−GF, closed bars). Roots were in non-saline nutrient solution. Letters denote significant difference between means using a *post hoc* Tukey's multiple comparisons test ($P < 0.05$). Two-way salt × GF ANOVA showed significant salt × GF interaction ($P = 0.0014$). Values are means (±SE, $n = 4$).

When submerged in non-saline water, leaf tissue ions remained similar to initial levels, the only exception being Cl^- in +GF plants, where at the end of the treatment the tissue ions had increased almost twofold above the initial values. Hence, for plants submerged in non-saline water, −GF resulted in significantly lower tissue Cl^- in leaves. For control plants with shoots in air, leaf ion concentrations remained at initial values throughout the experiment (see caption of Fig. 2).

In conclusion, leaf gas films were present for at least 5 d (saline) and 9 d (non-saline) submergence and significantly delayed Na^+ uptake, but not that of Cl^-, and also did not prevent substantial K^+ loss during submergence in saline water. In the following sections, we describe the effects of NaCl and submergence on the other physiological parameters measured for leaves and on plant growth and recovery upon desubmergence.

Removal of leaf gas films accelerates chlorophyll$_a$ degradation

Leaf chlorophyll$_a$ declined several days earlier when submerged plants had their gas films removed (Fig. 1). For

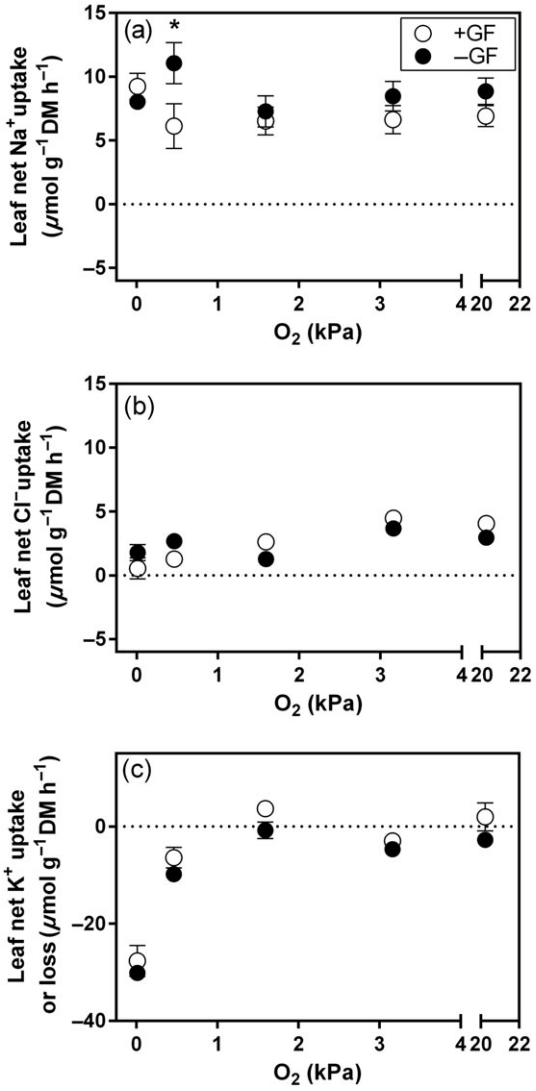

Figure 7. Leaf Na^+ (a), Cl^- (b) and K^+ (c) net uptake or loss of a youngest fully expanded leaf of rice submerged in 50 mM NaCl (the solution also contained basal ions, see Methods) for 24 h in the dark with gas film (+GF, open symbols) or treated with 0.1% Triton X-100 and without gas films (−GF, closed symbols). During incubation, the submergence solution was maintained at 0.01, 0.46, 1.59, 3.16 and 20.23 kPa O_2. * denotes significant difference between mean leaf ion uptake rates (Sidak's multiple comparisons test, $P < 0.05$). Two-way GF × pO_2 ANOVA showed a significant effect of GF on leaf Na^+ ($P = 0.0345$) and K^+ ($P = 0.0091$) uptake, although the overall effect was relatively small and on most time points not significant. pO_2 had a significant effect on K^+ loss ($P < 0.0001$), as ion loss increased severely when pO_2 was below 1.6 kPa. For Cl^-, two-way ANOVA showed a significant GF × pO_2 interaction ($P = 0.0050$). Values are means (±SE, $n = 4$). Tissue ion concentrations after 24 h incubation are displayed in Supporting Information Fig. S6.

example, after 5 d of submergence at 50 mM NaCl, −GF leaf chlorophyll$_a$ declined to 33% of initial levels, while plants retaining a gas film increased in chlorophyll by 28%. For plants initially with gas films, loss of gas films after 9 d of submergence was followed by declines in chlorophyll$_a$ levels to as low as 21% of initials after 16 d of submergence. The 50 mM NaCl decreased leaf chlorophyll$_a$ even further, as evident from a significant time × salt effect in both gas film treatments. However, when comparing single time points, the effect of salt was not substantial and with one exception (day 2, +GF), remained statistically insignificant in *post hoc* tests.

In spite of significant negative (Na^+, Cl^-) and positive (K^+) correlations found when plotting chlorophyll$_a$ concentrations against tissue ions concentrations (Supporting Information Fig. S4), a causal relationship between these two factors is not necessarily present, because of the different time points at which leaves were sampled. For example, leaf chlorophyll$_a$ declined with time of submergence regardless of salinity treatment (Fig. 1); this decline with time could then have resulted in lower chlorophyll$_a$ at later time points (where leaf Na^+ and Cl^- were at their highest in the saline treatment). We therefore suggest that chlorophyll$_a$ degradation is mainly caused by the effects of submergence *per se*, as also prevailing during non-saline submergence (see Discussion).

Leaf porosity is affected by salinity and gas film removal

Gas film removal caused leaf porosity to decline substantially within the first 5 d of submergence (mean tissue porosity of 2.0% after 5 d in non-saline water; Fig. 1), compared with leaves with intact gas films under the same conditions (mean tissue porosity of 10.4%, same as initials). The adverse effects of gas film removal were stronger for plants in saline than in non-saline solution (0.0 and 11.7% porosity in −GF and +GF plants on day 5, respectively); therefore, two-way ANOVA (time × salt) showed a significant salt effect for −GF plants only. Thus, +GF plants maintained higher leaf porosity during submergence until the gas films were lost naturally.

Gas films enhance underwater gas exchange

Gas films enhanced underwater P_N at 200 μM CO_2 (Fig. 3). At this CO_2 concentration, P_N rates of −GF leaves were 15% of +GF leaves (when first submerged in non-saline water). The positive effect of leaf gas films on underwater P_N was maintained for 5 d and then declined by day 9 as gas films diminished, with P_N of +GF plants reduced to 16% (non-saline) and 7% (saline) of initials on day 16. Results of three-way ANOVA (time × salt × GF) reflected this decrease over time with significant time × gas film interactions at both 200 and 2500 μM CO_2. Elevating CO_2 to 2500 μM closed the gap between +GF and −GF leaves, as now, gas film removal only reduced P_N to 72% of leaves with intact gas films. Increasing external CO_2 partially alleviated the negative effect of not possessing gas films on CO_2 uptake, that is, increasing external CO_2 can overcome the higher resistance of CO_2 uptake in leaves with no gas films. Plotting P_N against gas film thickness (Fig. 4) revealed significant positive correlations both at low ($r = 0.73$) and high ($r = 0.75$) CO_2, underlining the positive effect of gas films on underwater P_N.

The NaCl treatment only affected P_N significantly at high CO_2 and when gas films were removed. Under these

conditions, P_N rates were 25% lower on average when subject to NaCl (significant on days 1, 5 and 9). Thus, three-way ANOVA (time × salt × GF) performed at both high and low CO_2 only showed a significant salt effect at high CO_2. When plotting P_N against tissue ion concentrations (Supporting Information Fig. S5), and excluding from the analysis late time points where leaves were severely damaged by submergence (characterized by low leaf chlorophyll and low leaf porosity values, see caption in Supporting Information Fig. S5), only tissue Na^+ and P_N at 2500 μM CO_2 showed a significant negative correlation ($r = -0.61$). As no significant NaCl effect was detected for P_N at 200 μM CO_2 ($P \geq 0.2094$), the adverse impact on growth of submergence alone was significant whereas the 50 mM NaCl treatment during submergence had little additional effect on growth (see next section on growth analysis) in spite of P_N at high CO_2 revealing some damage to the photosynthetic apparatus.

Gas film removal significantly reduces rice growth when submerged

Rice shoots elongated during 9 d of submergence and were 52% longer than controls in air (average of NaCl and GF treatments, Table S1). Meanwhile, submergence severely inhibited tillering, as mean tiller numbers after 9 d of submergence across NaCl and GF treatments were only 1–1.75 in submerged plants compared with 4–4.5 in controls with shoots in air (Table S1).

Complete submergence over 9 d reduced root RGR more than the reduction in shoot RGR, and gas film removal caused further reductions to growth of plants when submerged in either non-saline or saline water (Fig. 5). During 9 d of submergence in non-saline water, +GF plants maintained root and shoot RGR at 27 and 61% of controls in air, respectively, while removal of gas film resulted in root and shoot RGR of 10 and 45% of controls in air. Plants that had gas films removed also showed reduced RGR during the recovery period after desubmergence (root and shoot RGR to 60 and 55% of controls in air). By contrast, plants with intact gas films had root and shoot RGR of 98 and 84% when desubmerged relative to controls in air.

NaCl had a tendency to further decrease RGR in −GF plants (e.g. root RGR during submergence in saline water; Fig. 5a), but two-way ANOVA (salt × GF) only detected significant gas film and no salinity effects during submergence and recovery. Nonetheless, a significant salt × GF interaction was found when analysing the amount of shoot tissue (% of total shoot DM) that had senesced and was scored as dead after the recovery period (Fig. 6), that is, plants subjected to both dilute Triton X-100 brushing and 50 mM NaCl had lower functioning leaf area for further growth than plants only subjected to one of these treatments.

In conclusion, gas film removal had a profound effect on rice RGR during complete submergence in non-saline water and the following recovery period. On the other hand, 50 mM NaCl in the submergence solution only had limited additional effect on growth.

Experiment 2 – the effect of pO_2 on leaf ion concentrations

To separate the effects of gas films acting as a possible physical barrier to ion uptake/loss, and gas films resulting in higher leaf O_2 status that could affect energy status and thus energy-dependent ion transport (potentially impacting K^+ retention and Na^+ and Cl^- 'exclusion' from leaves), excised leaves with and without gas films were incubated in submergence solution with 50 mM NaCl and pO_2 ranging from 0.01 to 20.3 kPa (Fig. 7). Low pO_2 did not seem to diminish gas films as clearly visible gas films were observed on all +GF leaves following the 24 h incubation.

Gas film removal resulted in 12% higher final tissue Na^+ concentration across the pO_2 range tested (Supporting Information Fig. S6); however, the difference was only significant at 0.46 kPa O_2. The little difference between +GF and −GF leaf Na^+ concentration was due to a high Na^+ influx in both cases: average net Na^+ uptake across the pO_2 range tested was 7.0 μmol Na^+ g^{-1} DM h^{-1} in +GF and 8.7 μmol Na^+ g^{-1} DM h^{-1} in −GF leaves (Fig. 7), so the leaf gas films acted only as a weak physical barrier as Na^+ entry into +GF leaves was substantial.

Gas films on leaves acting as a rather weak barrier to tissue ion fluxes were confirmed by changes in tissue K^+. Lowering pO_2 to 0.01 kPa resulted in a severe loss of tissue K^+ regardless of gas film presence (Fig. 7), with tissue K^+ decreasing to 21% (−GF) and 25% (+GF) of leaves incubated at 20.3 kPa O_2. Nonetheless, gas films did have a significant overall effect on tissue K^+ according to two-way ANOVA (see caption of Fig. 7).

Interestingly, leaf Cl^- showed a significant GF × pO_2 interaction, as removal of gas films decreased tissue Cl^- by 8–16% relative to leaves retaining a gas film at 1.59–20.23 kPa O_2, and increased tissue Cl^- by 20% at 0.01 and 0.46 kPa (Supporting Information Fig. S6). Although these differences in Cl^- concentrations at single pO_2 levels were not significant in *post hoc* tests, it should be noted that in experiment 1, presence of gas films also tended to result in higher tissue Cl^- on days 5 and 9 in both non-saline (significant on day 9) and saline (not significant) water compared with leaves without gas films. These coinciding observations from two separate experiments seem to indicate a complex interaction between leaf gas films and Cl^- uptake and resulting tissue concentrations.

In conclusion, this second experiment with varying pO_2 confirmed that gas films on leaves of rice apparently only act as a weak physical barrier to ion uptake/loss; a leaf-water interface, allowing for substantial K^+ net loss and Na^+ net uptake must have been present during the 24 h of submergence, despite the gas films being visibly present.

DISCUSSION

Gas film presence had the expected beneficial effects on plant growth, P_N, leaf chlorophyll$_a$, leaf porosity and shoot tissue survival for rice during submergence in saline (50 mM NaCl) water. However, the results did not support our initial hypothesis of rice leaf gas films acting as a strong physical barrier to ion uptake (Na^+, Cl^-) or loss (K^+) during

submergence in saline water. Although gas film removal significantly increased Na^+ uptake by plants submerged in artificial floodwater containing 50 mM NaCl, Na^+ and Cl^- accumulation and K^+ loss were substantial even in leaves possessing gas films (Fig. 2). Gas films acting as a rather weak physical barrier to Na^+ entry were confirmed in a separate experiment (Fig. 7 and Supporting Information Fig. S6), where removal of gas films resulted in a 12% increase in leaf Na^+ concentration.

Gas film retention time in the present experiment was up to 6 d longer than in the field (Winkel *et al.* 2014), indicating that turbid floodwaters may accelerate gas film loss. Interestingly, the gas films were retained longer by plants in the non-saline treatment as compared with those in the saline submergence (Fig. 1). Loss of gas films with time of submergence was not associated with clear structural changes of the surface of the leaf cuticle, except for wax platelets on papillae appearing more rounded (Supporting Information Figs S1 and S2). This loss of gas films without significant changes in cuticle surface structure was unexpected as leaf hydrophobicity is known to be related to the amount of wax platelets on the cuticle surface (see Neinhuis and Barthlott, 1997 for characterization of leaf hydrophobicity and the relationship with cuticle nanostructure). The recent description of the gene '*OsHSD1*' responsible for synthesis of rice epicuticular wax compounds (Zhang *et al.* 2016) adds new perspectives to further explore gas film retention during submergence.

Uptake of Na^+ and Cl^- and loss of K^+ indicates leaf-to-water contact even with gas films present

Leaves of rice with gas films when submerged in 50 mM NaCl for 9 d had tissue ion concentrations (μmol g^{-1} fresh mass) of Na^+ 145, Cl^- 106 and K^+ 38 (data not shown), respectively, which compare with maximum concentrations of Na^+ 94 and Cl^- 141 and minimum K^+ 52 in the coleoptiles of rice seedlings submerged for 42–186 h at 50 mM NaCl (Kurniasih *et al.* 2013). The coleoptiles emerged from seeds under water and lacked gas films (Kurniasih *et al.* 2013). The substantial entries of Na^+ and Cl^- for rice leaves with gas films when submerged in saline water at 72 and 93% of those for leaves without gas films (Fig. 2) contrast with the reduced ion entry into leaves of *M. siculus* with gas films of only 51 and 44% of the amounts without gas films (during the first 24 h of complete submergence, Teakle *et al.* 2014). These species differences in ion entry could be due to contrasting leaf morphology resulting in distinct three-dimensional (3D) structures of the gas films. The 3D tomograms of submerged *Spartina anglica* leaves with gas films indicated that along the leaf ridges, approximately 20% of the ridge surface is in direct contact with water (Lauridsen *et al.* 2014). Rice also possesses plicate leaves (Wu *et al.* 2011) where the majority of the external gas volume is present in the deep grooves between each ridge running parallel along leaves, and presumably areas along the ridges of leaves of rice must also have some direct contact with the floodwater. With time of submergence, and with possible declines in surface hydrophobicity, these exposed patches are likely to grow in size resulting in increasingly larger interfaces (i.e. areas of direct contact) between floodwater and the leaf surface. In contrast to rice and *S. anglica*, *M. siculus* does not possess plicate leaves (see photo of submerged leaf in Teakle *et al.* 2014), likely resulting in much less variation in 3D structure (and thickness) of the gas film across the leaf surface. Consequently, we suggest that the differences in ion entry observed between rice (this study) and *M. siculus* (Teakle *et al.* 2014) are due to 3D structural differences in the gas layer forming the interface between cuticle and floodwater, with likely more direct leaf-to-water contact in submerged rice than in *M. siculus*.

Presence of significant direct leaf-to-water contact for rice leaves with gas films was supported by substantial loss of K^+ from leaves submerged in water at low pO_2 (Fig. 7). K^+ loss during severe hypoxia or anoxia is, in the short-term, caused by depolarization of plasma membranes (Buwalda *et al.* 1988), leading to opening of voltage-gated ion channels (Ward *et al.* 2009), and during longer periods can result from deterioration or damage to membranes, as described for wheat roots (Buwalda *et al.* 1988; Greenway *et al.* 1992; Goggin & Colmer 2007). Meanwhile, the leaf K^+ loss observed when submerged in saline solution at air-equilibrium pO_2 (Figs 2 and 7) is most likely caused by high external Na^+ known to induce K^+ efflux (Shabala *et al.* 2006; Britto *et al.* 2010).

While removal of leaf gas films increased Na^+ uptake by leaves of rice submerged at 50 mM NaCl, Cl^- uptake was much more similar in −GF and +GF leaves (Fig. 2). In addition, on some occasions (submerged in non-saline water in experiment 1 and at high pO_2 in experiment 2), tissue Cl^- concentration was higher in +GF than in −GF leaves. We suggest that this difference in tissue Na^+ and Cl^- concentrations could be caused by these ions entering the leaf in different ways: Na^+ is likely to enter leaves down an electrochemical gradient, while Cl^- has to be actively taken up because of its negative charge. Rice coleoptiles submerged in 50 mM NaCl showed peak uptake of Cl^- during the initial 42–114 h of submergence (Kurniasih *et al.* 2013), and such Cl^- uptake can be a more rapid and less energy-demanding means to maintain cell turgor or volume than production of organic solutes (Raven 1985; Oren 1999). Energy available for Cl^- influxes via H^+–Cl^- symports and associated H^+ATPase activity required to maintain the H^+ gradient across the plasma membrane (Teakle & Tyerman 2010) is likely to be higher in +GF leaves because of higher sugar levels and O_2 uptake compared with −GF leaves (Pedersen *et al.* 2009; Winkel *et al.* 2013). The significant $pO_2 \times GF$ interaction on leaf Cl^- concentrations in experiment 2 further supports that Cl^- uptake is altered by leaf energy status. Indeed, ion net fluxes even in the anoxia-tolerant coleoptile of rice seedlings are substantially reduced in anoxia as compared with aerated conditions for seedlings submerged in 50 mM NaCl (Kurniasih *et al.* 2016). Tracer experiments are needed to separate the roles of ion influx or efflux on changes in net uptake rates and leaf ion concentrations of submerged rice.

Rice subjected to 50 mM NaCl in the root medium but with shoots in air in several earlier experiments accumulated higher leaf tissue Na^+ and Cl^- concentrations (Yeo & Flowers 1982; 1984; 1985; 1986) than those in the shoot tissues of rice during complete submergence in 50 mM NaCl (present study and

Kurniasih *et al.* 2013), possibly because of continuous ion transport to leaves via the transpiration stream of plants with shoots in air. Rice with roots in 50 mM NaCl and with shoots in air contained leaf Na^+ at 1996–2280 and Cl^- at 1770–2122 μmol g^{-1} DM after 7–10 d of treatment (Yeo & Flowers 1984; 1985), compared with the leaf Na^+ at 980 and Cl^- at 719 μmol g^{-1} DM in +GF plants after 9 d submergence in this present experiment. In some experiments with roots of rice in saline solutions and with shoots in air, leaf (Yeo *et al.* 1999) and shoot (Flowers & Yeo 1981; Yeo & Flowers 1984; 1986; Yeo *et al.* 1999; Kavitha *et al.* 2012) Na^+ and Cl^- concentrations were similar to concentrations in the present study. Differences in shoot Na^+ concentration between rice plants in different experiments can result from variations in Na^+ 'exclusion' ability amongst genotypes (Yeo & Flowers 1986; Yeo *et al.* 1999), relative humidity (Yeo & Flowers 1984), leaf age (Yeo *et al.* 1985) and treatment duration (Flowers & Yeo 1981).

Gas films enhance underwater P_N and delay leaf tissue degradation of submerged rice

Leaf gas films have beneficial effects on underwater P_N of rice both in non-saline (Pedersen *et al.* 2009; Winkel *et al.* 2014) and saline (present study, Fig. 3) submergence. 3D diffusion modelling has demonstrated the enhanced leaf-floodwater gas exchange by leaves with gas films if stomata remain at least partially open during submergence (Verboven *et al.* 2014). The significant adverse effect of gas film removal on growth (Fig. 5) is in accordance with a previous study of submerged rice (Pedersen *et al.* 2009).

In addition to lowering P_N, removal of leaf gas films leads to earlier leaf $chlorophyll_a$ degradation of submerged leaves (Fig. 1). This contrasts with a previous study where no difference in total leaf chlorophyll between +GF and –GF plants was found during 7 d submergence in the field (Winkel *et al.* 2013). We suggest that the lack of decline in $chlorophyll_a$ in −GF plants could be due to Winkel *et al.* (2013) sampling the youngest fully developed leaf at all time points and not beyond 7 d. In a subsequent experiment, Winkel *et al.* (2014) observed no chlorophyll decline until day 7 (second youngest fully expanded leaf, submerged for 13 d in the field, +GF only) consistent with declines after 9 d in the present study. Yeo and Flowers (1983) established the leaf Na^+ concentrations associated with a 50% loss of chlorophyll (LC_{50}) for nine rice genotypes with shoots in air. However, for plants in the present study, $chlorophyll_a$ degradation was mainly caused by duration of submergence (explaining 76% of the variation in $chlorophyll_a$ according to ANOVA) rather than leaf Na^+ concentration (5% of the variation), and $chlorophyll_a$ degradation was severe even in leaves of plants submerged in non-saline water, so we refrained from calculating a LC_{50} in the present study. Leaf senescence is a common feature of submerged rice and has been associated with the accumulation of ethylene causing chlorophyll degradation (Jackson *et al.* 1987; Ella *et al.* 2003).

Leaf hydrophobicity has previously been suggested as an adaptation to prevent adverse effects of salt spray on leaves of some coastal plants (Ahmad & Wainwright 1976; McNeilly *et al.* 1987). Variation in leaf wettability and leaf Na^+ retention (upon spraying with or immersion into water containing 500 mM NaCl) was linked to distributions of three *Agrostis stolinefera* ecotypes growing in sheltered inland habitats, seawater spray-zone or salt marshes (Ahmad & Wainwright 1976). The inland ecotype showed high wettability because of lower contact angles and shorter epicuticular waxes than the ecotypes in the salt-spray and salt marsh zones, resulting in 16 times higher Na^+ retention on the surface of leaves after 5 s immersion into saline water. Ahmad and Wainwright (1976) suggested that differences in adaxial and abaxial sides for leaf hydrophobicity in spray-zone plants but not in salt marsh plants could be an adaption to episodic inundations of the marsh plants, as inundation would affect both sides of the leaf in contrast to salt-spray. However, another low salt marsh plant from the intertidal zone (*S. anglica*) being hydrophobic only on the adaxial leaf side (Winkel *et al.* 2011) is not in support of two-sided leaf hydrophobicity as a general adaptation to salt water submergence, but like for rice with two-sided leaf gas films (Winkel *et al.* 2013), the one-sided leaf gas films on submerged *S. anglica* also benefit internal O_2 status both during the day and at night.

CONCLUSIONS

Leaf gas films contribute to rice submergence tolerance by improving underwater gas exchange, growth, internal aeration and plant sugar levels (Pedersen *et al.* 2009; Winkel *et al.* 2013). This study found that during submergence in saline water (50 mM NaCl), gas films diminished earlier than for leaves in freshwater and that rice plants possessing leaf gas films maintained higher levels of underwater P_N, more growth during submergence and recovery, greater proportion of surviving shoot biomass and better maintained leaf porosity and $chlorophyll_a$. Submergence was more detrimental to leaf P_N than the additional effect of 50 mM NaCl. However, gas films on leaves of rice delayed Na^+ entry to a much smaller degree compared with leaves of *M. siculus*, which was likely due to 3D structural differences in the gas layers on these two species with probable greater leaf-to-water contact for rice. Rice has plicate leaves, like *S. anglica*, for which the ridges have some direct contact with surrounding water even when the gas film is present (Lauridsen *et al.* 2014). Varying pO_2 had no effect on leaf Na^+ net uptake, suggesting that the observed delay of Na^+ uptake in +GF leaves should be attributed to gas films acting as a physical barrier rather than from the possible influence of altered O_2 supply and potential improved leaf energy status.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Scanning electron microscopy micrographs of leaf surfaces of rice subject to 0–16 d submergence in non-saline (0 mM NaCl) or saline (50 mM NaCl) water (containing basal ions see Methods). Each leaf is shown at 7000 × magnification (left) showing stomata horizontal field width = 36.6 μm and 500 × magnification (right) horizontal field width = 512 μm.

Figure S2. Scanning electron microscopy micrographs showing wax platelets on leaf surface after loss of leaf hydrophobicity and leaf gas film disappearance (a, plants had been submerged in water with 50 mM NaCl for 9 d), and wax platelets on papillae before (b, prior to submergence) and after (c, plants had been submerged in water with 50 mM NaCl for 9 d) loss of leaf hydrophobicity and leaf gas film disappearance. Leaves are shown at 27.000 × (a) and 45.000 × (b, c) magnification (horizontal field width = 3.9 and 2.5 μm, respectively).

Figure S3. Leaf Na^+ (a), Cl^- (b) and K^+ (c) concentrations in the tissue water (mM) with time of submergence in water (containing basal ions, see Methods) with 0 mM NaCl (squares) or 50 mM NaCl (circles) for rice plants with leaf gas films (+GF, open symbols) or treated with 0.1% Triton X-100 and without gas films (−GF, closed symbols). Samples were the entire third and part of the fourth leaf (see Methods). Roots were in non-saline nutrient solution. Values are means [±SE, $n = 3$–4 except Na^+ on day 5 at 0 mM NaCl (−GF) where $n = 1$ due to a sampling error].

Figure S4. Correlations among leaf Na^+ (a), Cl^- (b) and K^+ (c) concentrations (data from Fig. 2) and corresponding leaf chlorophyll$_a$ concentrations (data from Fig. 1b) after submergence of rice in water (containing basal ions, see Methods) with 50 mM NaCl. Leaves were either left untreated, thus retaining a leaf gas film (+GF, open symbols) or treated with 0.1% Triton X-100 (−GF, closed symbols). Roots were in non-saline nutrient solution. r values from non-parametric Spearman rank correlation analysis, * denoting levels of significance (levels of $P > 0.05$, $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$ or $P \leq 0.0001$ are denoted by n.s., *, **, ***, ****, respectively): Na^+ $r = -0.5084$**; Cl^- $r = -0.3658$*; K^+ $r = 0.4664$**.

Figure S5. Correlations among leaf Na^+ (a), Cl^- (b) and K^+ (c) concentrations (data from Fig. 2) with corresponding underwater P_N (μmol O_2 m^{-2} s^{-1}) at 2500 μM CO_2 and 50 mM NaCl (data from Fig. 3b). Leaves were either left untreated, thus retaining a leaf gas film (+GF) or treated with 0.1% Triton X-100 (−GF). Roots were in non-saline nutrient solution, and shoots were submerged in water containing NaCl treatments and basal ions (see Methods). r values from non-parametric Spearman rank correlation analysis, * denoting levels of significance (levels of $P > 0.05$, $P \leq 0.05$, $P \leq 0.01$, $P \leq 0.001$ or $P \leq 0.0001$ are denoted by n.s., *, **, ***, ****, respectively): Na^+ $r = -0.6078$**; Cl^- $r = -0.3243$ n.s.; K^+ $r = 0.1923$ n.s. † denotes points excluded from the correlation analysis to prevent leaf deterioration with time of submergence to draw the correlation. Points were excluded when leaf both porosity and chlorophyll$_a$ was <4.5% and 8.3 mg g^{-1} DM, respectively, as for day 16 (+GF) and days 5 and 9 (−GF). At 200 μM, free CO_2 all correlations were not significant (Spearman rank correlation analysis, $P > 0.05$, data not shown).

Figure S6. Leaf Na^+ (a), Cl^- (b) and K^+ (c) concentrations of a youngest fully expanded leaf of rice submerged in 50 mM NaCl (containing also basal ions, see Methods) for 24 h in the dark with gas films (+GF, open symbols) or treated with 0.1% Triton X-100 and without gas films (−GF, closed symbols). During incubation, the submergence solution was maintained at 0.01, 0.46, 1.59, 3.16 and 20.23 kPa O_2. * denotes significant difference between ion concentrations (Sidak's multiple comparisons test, $P < 0.05$). Two-way GF × pO_2 ANOVA showed a significant effect of GF on leaf Na^+ ($P = 0.0345$) and K^+ concentrations ($P = 0.0091$). pO_2 had a significant effect on K^+ concentrations ($P < 0.0001$). For Cl^-, two-way ANOVA showed a significant pO_2 × GF interaction ($P = 0.0050$). Values are means (±SE, $n = 4$).

Table S1. Shoot length and number of tillers of rice plants submerged in water (containing basal ions, see Methods) with 0 mM NaCl or 50 mM NaCl with leaf gas films (+GF) or treated with 0.1% Triton X-100 and without gas films (−GF). +GF and –GF plants are from different batches; hence, these have separate emergent (shoots in air) controls. Roots were in non-saline nutrient solution. Letters denote significant difference ($P < 0.05$) between means (±SE, $n = 4$) according to one-way ANOVA with Sidak's multiple comparisons *post hoc* test (shoot length) or non-parametric Kruskal–Wallis with Dunn's multiple comparisons test (number of tillers).

