2014

Leaf gas films delay salt entry and enhance underwater photosynthesis and internal aeration of Melilotus siculus submerged in saline water

Natasha Lea Teakle
Edith Cowan University, n.ayers@ecu.edu.au

Timothy David Colmer

Ole Pedersen

10.1111/pce.12269

Leaf gas films delay salt entry and enhance underwater photosynthesis and internal aeration of *Melilotus siculus* submerged in saline water

Natasha L. Teakle[1,2,3], Timothy D. Colmer[1] and Ole Pedersen [1,4,5]

[1] School of Plant Biology (M084) and UWA Institute of Agriculture, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

[2] Centre for Ecohydrology, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

[3] Graduate Research School, Edith Cowan University, 270 Joondalup Dr, Joondalup, WA 6027, Australia

[4] Institute of Advanced Studies, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

[5] Freshwater Biological Laboratory, University of Copenhagen, Universitetsparken 4, 3rd floor, 2100 Copenhagen, Denmark

Corresponding author: Ole Pedersen, opedersen@bio.ku.dk

Abstract

A combination of flooding and salinity is detrimental to most plants. We studied tolerance of complete submergence in saline water for *Melilotus siculus*, an annual legume with superhydrophobic leaf surfaces that retain gas films when under water. *M. siculus* survived complete submergence of one week at low salinity (up to 50 mol m^{-3} NaCl), but did not recover following de-submergence from 100 mol m^{-3} NaCl. The leaf gas films protected against direct salt ingress into the leaves when submerged in saline water, enabling underwater photosynthesis even after 3 d of complete submergence. By contrast, leaves with the gas films experimentally removed suffered from substantial Na^+ and Cl^- intrusion and lost the capacity for underwater photosynthesis. Similarly, plants in saline water and without gas films lost more K^+ than those with intact gas films. This study has demonstrated that leaf gas films reduce Na^+ and Cl^- ingress into leaves when submerged by saline water – the thin gas layer physically separates the floodwater from the leaf surface. This feature aids survival of plants exposed to short-term saline submergence, as well as the previously recognised beneficial effects of gas exchange under water.

Keywords

aerenchyma; *Melilotus siculus*; flooding tolerance; salinity tolerance; salt intrusion; leaf Na^+; leaf K^+; leaf Cl^-; legume; plant submergence tolerance; underwater photosynthesis

Introduction

Flooding can be a severe abiotic stress on plants (Bailey-Serres & Voesenek 2008) and in various situations the water can be saline. Flooded soils are typically low in O_2 and when shoots are submerged plants face further restrictions on their gas exchange (Armstrong 1979; Voesenek *et al.* 2006). Traits associated with plant flooding tolerance include aerenchyma for internal aeration (Armstrong 1979), adventitious roots (Jackson & Drew 1984), anoxia tolerance in some tissues (Greenway & Gibbs 2003), shoot elongation response (Bailey-Serres & Voesenek 2008), and capacity for underwater photosynthesis (Colmer *et al.* 2011). Flooding regimes of different depths and durations, however, impose selection pressures for various combinations of these and other traits in wetland plants (Colmer & Voesenek 2009).

Salt tolerance is generally associated with the ability to regulate Na^+, K^+ and Cl^- transport to the shoots (Plett & Møller 2010; Teakle & Tyerman 2010; Shabala & Mackay 2011) and effective ion compartmentation and maintenance of favourable water relations (reviewed by Flowers & Colmer 2008; Munns & Tester 2008). When combined with salinity, the low O_2 associated with flooding impacts on the energetic demands of regulating ion transport to prevent shoot Na^+ and Cl^- accumulating to toxic levels (Barrett-Lennard 2003; Colmer & Flowers 2008) and to maintain sufficient K^+ (Barrett-Lennard & Shabala 2013). Surprisingly, very few studies have evaluated the mechanisms of plant tolerance to saline submergence (e.g., in halophytes reviewed by Colmer & Flowers 2008).

Melilotus siculus (Turra) B.D. Jacks. (syn. *Melilotus messanensis*) is a waterlogging- and salt-tolerant annual legume species (Marañòn *et al.* 1989; Rogers *et al.* 2008; Teakle *et al.* 2012) used for pasture on some soils/small areas in some regions with Mediterranean climates. Waterlogging tolerance of *M. siculus* is linked to a high capacity for internal root aeration via aerenchymatous phellem (up to 50% porosity, Verboven *et al.* 2012) and formation of numerous new lateral roots of high porosity (Teakle *et al.* 2011). This internal supply of O_2 for respiration provides energy to regulate root ion transport under combined waterlogging and salinity (Teakle *et al.* 2012). Areas naturally inhabited by *M. siculus* (Marañòn *et al.* 1989) or saline agricultural land being targeted for pasture production (Bonython *et al.* 2011) can also experience short-term flooding, resulting in plant submergence. Tolerance of *M. siculus* to submergence has not been previously studied, although the leaf surfaces were observed to possess gas films when submerged at a saline field site

(NL Teakle, *personal observation*). Several terrestrial wetland plants possess superhydrophobic leaves that retain a thin gas film when submerged (Raskin & Kende 1983; Colmer & Pedersen 2008b). Superhydrophobicity of leaves is normally associated with the nano-structure of the cuticle and water repellent surfaces of leaves promote 'self cleansing', enhancing leaf performance and reputably lowering susceptibility to pathogens (Neinhuis & Barthlott 1997). Experiments with other wetland species have demonstrated that leaf gas films enhance underwater photosynthesis (Raskin & Kende 1983; Colmer & Pedersen 2008b) and also whole plant internal aeration (Pedersen *et al.* 2009; Winkel *et al.* 2011; Winkel *et al.* 2013), thus contributing to submergence tolerance. However, the role of leaf gas films in tolerance to saline submergence has not previously been studied.

The present study assessed the tolerance of *M. siculus* to submergence in non-saline and saline conditions. Responses of growth and tissue ion concentrations to increasing external NaCl were established for plants when roots were waterlogged and also when the shoots were completely submerged. The submerged leaves of *M. siculus* possessed gas films (Fig. 1). Since gas films prevent direct contact of the water with the underlying tissue surface, we hypothesised that this feature can 'protect' leaves from Na^+ and Cl^- intrusion and thus enhance survival during submergence in saline water. We also hypothesised that leaf gas films of *M. siculus* improve underwater gas exchange and internal aeration, as also described above for other species with gas films on submerged leaves.

Materials and Methods

Plant culture

Seeds of *Melilotus siculus* (SARDI 36983) were scarified, washed in 0.04% NaHClO, rinsed thoroughly in deionised (DI) water and then imbibed in aerated 0.5 mol m^{-3} $CaSO_4$ in darkness for 3 h before being transferred to mesh over aerated 10%-strength aerated nutrient solution. After 3 d, seedlings were transferred to 25%-strength aerated nutrient solution and exposed to light. Seven d after germination, seedlings were transplanted into plastic pots containing 4.5 l of 50%-strength aerated nutrient solution. There were 8 seedlings in each pot, held individually in holes in the pot lid using polyethylene foam. At 14 d after imbibition, solutions were changed to 100%-strength aerated nutrient solution. Nutrient solution at 100% concentration consisted of macronutrients (mol m^{-3}): 0.5 KH_2PO_4, 3.0 KNO_3, 4.0 $Ca(NO_3)_2$, 1.0 $MgSO_4$; and micronutrients (mmol m^{-3}): 37.5

$FeNa_3EDTA$, 23.0 H_3BO_3, 4.5 $MnCl_2$, 4.0 $ZnSO_4$, 1.5 $CuSO_4$, and 0.05 MoO_3, as used previously for this species (e.g., Rogers *et al.* 2008; Teakle *et al.* 2011). NaCl concentration was 0.1 mol m^{-3}. Solution pH was buffered with 2.5 mol m^{-3} MES (2-[N-Morpholino]ethanesulfonic acid) adjusted with KOH to pH 6.3. Nutrient solutions were aerated, changed weekly and topped up with DI water as required. All pots were covered with Al-foil to exclude light. Plants were kept for the duration of the experiment in a naturally lit, temperature controlled (20/15°C day/night) phytotron during September to October 2010 in Perth, Western Australia. Average photosynthetically active radiation (PAR) within the phytotron at midday during the experimental period was 1149 µmol m^{-2} s^{-1}.

Root-zone salinity and O_2 treatments

Salinity treatments in the root-zone medium were imposed 21 d after imbibition, by adding 25 mol m^{-3} NaCl increments every 12 h to reach the final concentrations of 25, 50 or 100 mol m^{-3}, also with control solutions maintained at 0.1 mol m^{-3} NaCl. Two days after the final NaCl concentrations were reached, a hypoxic pre-treatment was given to the root-zone medium of stagnant designated pots by bubbling with N_2 until the O_2 level was approximately 10% of that at air-equilibrium. After 24 h, the solutions in these pots were changed to a deoxygenated stagnant 0.1% (w/v) agar nutrient solution (Wiengweera *et al.* 1997), with the mineral composition as described above. Plants were then grown for an additional 3 d in this stagnant root-zone medium prior to imposition of the shoot submergence treatments.

Submergence treatment

For the submergence treatments, individual plants were carefully removed from the 4.5 l pots and the intact roots placed (with the same foam holder around the stem base) into a 250 ml black plastic bottle containing the same nutrient solution (with the same salinity level and also containing de-oxygenated stagnant agar solution) as for each particular plant in the various root-zone treatments described in the preceding section. Each bottle also contained 7 glass marbles to weigh the bottle down in the submergence tanks. The top of the bottle and foam were wrapped in parafilm, to impede the possibility of nutrients moving through the foam holder, which was acting like a plug in the bottle neck. The bottles containing one plant each were then transferred into clear Perspex cylinders filled with 12 l of solution (shoots completely submerged and unable to reach air). The submergence solution contained 2 mol m^{-3} $CaSO_4$, 0.25 mol m^{-3} $MgSO_4$ and 1 mol m^{-3} $KHCO_3$. Dissolved CO_2 was maintained at 140 mmol m^{-3} (with pH at 7.2) using a pressurised CO_2 cylinder

and bubble stone in the tanks and a pH controller (α-control, Dupla Aquaristik, Bielefeld, Germany). The submergence solution was circulated using a pump attached to a sponge filter and a UV filter. Four circulating lines were set-up, with 8 tanks per line and 6 plants per tank. Non-submerged plants were also transferred to 250 ml bottles containing glass marbles and the same nutrient solution with 0.1% (w/v) agar and the various NaCl treatments as used above, and placed in tanks filled with water to just below the top of the bottle (i.e. 'emergent' with shoots in air).

Response to submergence with different salinity levels

This dose-response experiment had an overall design of 4 NaCl treatments (0.1, 25, 50 and 100 mol m^{-3}) and 2 submergence treatments (fully submerged and shoots in air). Four submergence lines were set-up; one for each of the 4 salinity treatments (0.1, 25, 50 and 100 mol m^{-3} NaCl). Plants were completely submerged for 7 d. After 3 d submergence, one plant per treatment was harvested to measure underwater net photosynthesis of excised leaves. Ion concentrations and dry mass (DM) were measured after 7 d of submergence. After the 7 d submergence period, the remaining plants in each tank were removed (i.e. 'de-submerged') and recovery from submergence was assessed after a further 7 d. Details of measurements are given below and for the treatments in Table 1.

Role of leaf gas films in tolerance to saline submergence

Plants were grown as in the dose-response experiment (see above), but only with root-zone salt treatments of 0.1 and 100 mol m^{-3} NaCl 7 d prior to submergence with hypoxic-stagnant conditions also imposed for the final 4 d prior to submergence. The main experimental design (Treatments 1-8 in Table 1) was: 2 submergence treatments (fully submerged or shoots in air); 2 leaf gas film treatments (brushed with water, or brushed with 0.1% v/v Triton X-100 to remove gas films); 2 salinity treatments (0.1 or 100 mol m^{-3} NaCl). Emergent controls (shoots in air) with aerated roots (Treatments 9-12 in Table 1) were also included in this experiment.

The submergence treatments were imposed 28 d after imbibition. As indicated above in the experimental design, just prior to submergence, leaf gas films were removed from half of the plants by brushing both sides of the leaves with 0.1% v/v Triton X using a soft paintbrush, as in a previous study (Pedersen *et al.* 2009); in this earlier research on rice, daily brushing of leaves with this dilute Triton X for plants with the shoot in air did not significantly affect the growth over a 7 d period compared with non-brushed controls (Pedersen *et al.* 2009). After brushing of the leaves, the whole

shoot was then rinsed in submergence solution prior to placement into the tanks. Plants with gas films kept intact were brushed with DI water prior to placement into the tanks.

Plants were submerged (and subsequently sampled) during the afternoon as described for the dose response experiment. Individuals from each treatment were randomly harvested at daily intervals for various measurements outlined below, including tissue ion concentrations. After 6 d submergence, the remaining plants were de-submerged and recovery assessed after an additional 7 d.

Harvests and fresh and dry mass measurements

An initial harvest for root and shoot fresh mass (FM) and DM was taken 21 d after imbibition (when NaCl treatment commenced). Additional harvests were then taken at the time of submergence (28 d after imbibition, after the salt and stagnant root-zone pre-treatments) and at various subsequent intervals for the different experiments (see above and figure legends). Young, fully expanded leaves were sampled *in situ* for measurements of underwater net photosynthesis and tissue chlorophyll concentration (details below). Plants were then removed from the tanks and bottles, and roots were washed 3 times (for 10 s each time) in 4 mol m^{-3} $CaSO_4$ with mannitol at a concentration iso-osmotic to the root-zone NaCl treatment (Lang 1967). Roots were separated from shoots and maximum root length and FM were recorded. Shoots were rinsed in 2 mol m^{-3} $CaSO_4$ with mannitol at a concentration iso-osmotic to the submergence NaCl treatment and tissues collected for measurements of mass and ion concentrations. Total shoot FM was recorded and tissues were oven dried at 60°C for 3 d, after which DM was recorded and tissue ion concentrations were measured (see next section). Extra plants were used for measuring petiole and tap root porosity (described below).

Concentrations of tissue ions

Concentrations of Na^+, K^+ and Cl^- were measured in dried samples of leaflets or whole shoots. 100 mg of finely ground dried tissue was extracted in 10 ml of 0.5 M HNO_3 for 2 d at 30 °C (Munns *et al.* 2010). Extracts were measured for Na^+ and K^+ (Jenway PFP7 Flame Photometer, Essex UK) and Cl^- (Slamed Chloridometer CHL 50, Frankfurt Germany). The reliability of these analyses was confirmed by taking a reference plant sample (ASPAC #85) with known ionic composition through the same procedures.

Underwater net photosynthesis

The youngest, fully expanded leaves were removed from plants that had been submerged for 3 d. Underwater net photosynthesis (P_N) was measured essentially as described by Pedersen *et al.* (2013). Gas films were removed from half of the samples by brushing with 0.1% v/v Triton X and then rinsing using a solution of the same composition as the incubation medium. Leaves were carefully added to 25 mL glass vials containing incubation solution, which was the same as the submergence solution the plants had come from (i.e. with either 0.1 or 100 mol m^{-3} NaCl in the basal medium of 2.0 mol m^{-3} $CaSO_4$, 0.25 mol m^{-3} $MgSO_4$ and 1.0 mol m^{-3} $KHCO_3$) plus 5.0 mol m^{-3} TES (2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid) buffer to maintain the pH at 7.2 after adjustment with 0.5 M HCl, so that dissolved CO_2 was at 140 mmol m^{-3}. The pO_2 of the solution was adjusted to approximately 10 kPa by mixing equal volumes of N_2 or air-bubbled solution; starting with O_2 below air-equilibrium helps to prevent photorespiration (Setter *et al.* 1989; Pedersen *et al.* 2011). The top of the glass vials were sealed with Parafilm® and aluminium foil, instead of glass stoppers, to prevent the pressure that can occur from stopper insertion causing the gas film to 'collapse' on these leaves. Blank vials without leaves were also included. Vials were incubated on a rotating wheel within an illuminated (PAR = 700 µmol m^{-2} s^{-1}) water bath (20°C) for 60 min. Dissolved O_2 concentrations were then measured using a Clarke-type O_2 microelectrode (OX-25, Unisense A/S, Aarhus, Denmark). Immediately after these measurements, leaflet samples were weighed and leaflet area measured using a leaf area meter (Li-Cor LI-3000, Lincoln, USA).

Net photosynthesis for leaves of intact plants in air was also measured. An infra-red gas analyser (Li-Cor LI-6400) attached to a leaf cuvette was used to measure light-saturated net photosynthesis by young, fully expanded leaves at ambient CO_2 (390 µmol mol^{-1}) with PAR of 1500 µmol m^{-2} s^{-1} (maximum PAR in phytotron).

Porosity measurements

Porosity was measured on petioles and tap roots using the 'buoyancy method' (Raskin 1983; Thomson *et al.* 1990). Maximum root and stem diameters were measured with digital callipers before the plants were separated into roots and shoots at the hypocotyl. Whole petioles with young, fully expanded leaflets were removed and the length and FM of the petiole recorded. A minimum of

0.2 g FM of petiole tissue was used. Approximately 0.5 g FM of the upper part of the tap root containing phellem (Teakle *et al.* 2011) was used for root samples.

Chlorophyll analysis

Leaflet samples from plants that had been submerged for 3 d were frozen in liquid N_2 and freeze-dried. 20 mg of ground sample was extracted in 1.25 ml of cold 100% methanol for 30 min in darkness (Wellburn 1994). Samples were centrifuged for 10 min at 1000 rpm in a microcentrifuge at 4°C. The supernatant was removed and 2 µl analysed across 220 to 750 nm using a NanoDrop Spectrophotometer (ND 1000, Thermo Scientific, Asheville, USA). Based on the nanodrop resolution of 3 nm, the following equations from Wellburn (1994) were used to calculate chlorophylls *a* (Chl *a*) and *b* (Chl *b*) using absorbance (Abs) at 653 and 666 nm.

Equation 1: Chl $a = 15.65 \times Abs_{666} - 7.34 \times Abs_{653}$

Equation 2: Chl $b = 27.05 \times Abs_{653} - 11.21 \times Abs_{666}$

Internal pO_2 of petioles

Internal pO_2 at the distal end of petioles of excised leaves was measured when reliant on internal O_2 movement, to evaluate O_2 entry and supply via leaflets when in air (with the petiole in deoxygenated medium) or when submerged with or without leaf gas films. Each leaf with petiole (approximately 100 mm in length) was mounted on a stainless steel mesh in a trough following the procedure of Colmer & Pedersen (2008a). The petiole was immersed in 0.1% w/v deoxygenated agar prepared in the same submergence solution as above, whereas the leaf with its 3 leaflets protruded out of the trough (i.e. leaflets exposed to air). An O_2 microelectrode (tip diameter 25 µm, OX25, Unisense A/S, Denmark) connected to a pA meter (Multimeter, Unisense A/S, Denmark) was inserted 150 µm into the petiole, 50 mm below the leaflets, and pO_2 was followed over time. Petiole pO_2 was measured *i*) with the leaflets exposed to air, *ii*) with the leaflets submerged with intact gas films, *iii*) with the leaflets submerged but with the gas films removed (see above), and *iv*) with the leaflets severed. Measurements were taken at 20 °C in darkness.

Data analyses

A minimum of 4 replicates per treatment combination were used in all experiments. Tanks connected to circulation lines were blocked per salinity treatment and within each line of tanks the other treatments (submergence level, gas films) were randomly allocated to tanks. Data were

analysed using GraphPad Prism 6.0. Residuals were checked for normality and homogenous variance. Most data were normally distributed and analysed using one- or two-way ANOVA and treatment comparisons made using Tukeys multiple comparison test or Least Significant Difference (LSD). Shoot RGR (Fig. 6) data were not normally distributed so non-parametric tests were used. The Kruskal-Wallis analysis was used to test for overall significant differences and Dunn's post-hoc test to compare between treatments. Significance level of $P < 0.05$ was used for all analyses and 'n.s.' indicates non-significant.

Results

Response to submergence with different salinity levels

M. siculus survived 7 d of submergence, even with 100 mol m^{-3} NaCl, the highest salt level and longest duration tested. However, after de-submergence (with the various root-zone NaCl treatments maintained), the plants previously submerged in 100 mol m^{-3} NaCl subsequently died; whereas, plants previously submerged in 0.1, 25 or 50 mol m^{-3} NaCl survived.

Shoot DM after 7 d of submergence was 0.12 g per plant (average for all NaCl treatments, no significant effect of NaCl) whereas it was 0.46 g for plants with shoots in air (emergent; average for all NaCl treatments). This submergence effect on shoot DM resulted from the cessation of DM increments when submerged, whereas the emergent plants grew.

As hypothesised, shoot Na^+ and Cl^- concentrations increased with each higher NaCl treatment; the increases in concentrations of these ions in the shoots of submerged plants were far greater than those of emergent plants (Fig. 2). A dose-effect was also evident under both flooding conditions. For example, shoot Cl^- concentrations of emergent as well as completely submerged plants were 2.3-fold higher in plants exposed to high salt (100 mol m^{-3} NaCl) compared to those in low salt (25 mol m^{-3} NaCl) concentration. Shoot Na^+ concentration also showed a similar dose-dependent pattern for emergent and submerged plants, but again with higher overall concentrations in the submerged plants compared to plants with emergent shoots. Salt exposure reduced shoot K^+ concentrations in both emergent and submerged plants; shoot K^+ did not differ between emergent and submerged plants in 25 and 50 mol m^{-3} NaCl, but it was 34% less for submerged plants at 100 mol m^{-3} NaCl (Fig. 2c).

Leaf gas films – influence on leaf ion concentrations during submergence

Gas films were present on both sides of *M. siculus* leaves when under water (Fig. 1), but the films only persisted for 3 d. Here we describe the effect of gas film removal on tissue ions, and in the next section we present the effects on underwater net photosynthesis and internal aeration.

For plants with emergent shoots there was no effect of leaf brushing with 0.1% v/v Triton X (and rinsing) on tissue ion concentrations, but in both cases (i.e. the two types of plants with shoots in air) there was a steady decline in K^+ concentration in the leaves with time (Fig. 3).

For the submergence treatments, leaflet Na^+ and Cl^- concentrations had tripled within the first day of submergence in 100 mol m^{-3} NaCl (Fig. 3a,c), for plants with gas films removed (i.e. brushed with 0.1 % v/v Triton X and rinsed prior to submergence).Whereas, for plants with intact gas films there was only a ~10% increase in leaflet Na^+ and Cl^- concentrations in the first day after submergence in 100 mol m^{-3} NaCl (Fig. 3a,c). For these leaflets with gas films, tissue Na^+ and Cl^- also remained relatively low on the second day, but then increased substantially on the third and fourth days. Interestingly, these increases in tissue ions commencing on day 3 (Fig. 3a,c) coincided with a visual decline in the gas film presence. A similar, although less pronounced, effect of removal of gas films on ion entry was also measured in the whole shoot (Fig. 3b,d).

Gas film presence also influenced tissue K^+ concentration of submerged plants. Young leaflet and whole shoot K^+ concentrations declined soon after submergence when leaf gas films had been removed, resulting in lower tissue K^+ in submerged plants with gas films removed as compared with those when the films were intact. This difference was evident up to day 4, but by day 6 of submergence there was little difference between plants initially with (the gas films only persisted for 3 d) or without (i.e. artificially removed at the time of submergence) gas films (Fig. 3e,f).

Shoot net uptake of ions for the initial 24 h after submergence was substantially higher when the leaf gas films had been experimentally removed compared to plants with intact gas films (Fig. 3a-d). In the whole shoot, Na^+ increased 2.2-fold in plants without gas films *versus* 1.6-fold in those with gas films (Cl^-; 2.7 without *versus* 1.7 with gas films) and the ion ingress was even more pronounced in the youngest fully expanded leaflets where Na^+ increased 2.8-fold in tissues when

without gas films and by only 1.4-fold in those with gas films (Cl^-; 3.5 without *versus* 1.5 with gas films).

Leaf gas films – influence on underwater net photosynthesis and internal aeration

O_2 dynamics were evaluated in petioles when reliant on O_2 diffusion via the lamina (i.e. leaflet) surfaces. Leaves with petioles of ~ 100 mm length were excised and then the petiole portion was submerged in deoxygenated 0.1% agar submergence solution, all in darkness, so that the only source of O_2 would be via longitudinal internal diffusion. A microelectrode measured tissue O_2 at the distal end, initially with the lamina in air and then following lamina submergence, removal of leaf gas films, and finally lamina excision. As expected (see Introduction), O_2 status of the petiole declined upon submergence of the leaflets in water at air-equilibrium, and removal of gas films further restricted the supply of O_2 to the petiole (Fig. 4). Excision of the leaflets caused pO_2 to drop within the petiole to under 2 kPa (Fig. 4); then, entry of O_2 would have been only via the short stub of petiole and cut surfaces remaining in the water.

The enhancement of underwater gas exchange via gas films was clearly evident in measurements of underwater net photosynthesis (P_N) of individual leaflets; i.e. enhanced CO_2 uptake from water. Removal of the gas films from leaflets grown in air (and submerged for the first time) reduced the underwater P_N to 41% (non-saline) and 35% (saline), as compared to those with intact gas films (Fig. 5). Similarly, leaflets of plants that had been submerged for 3 d with no gas film showed a substantial decline in underwater P_N as compared to those with intact gas films (Fig. 5). There was a significant interaction between submergence and NaCl treatment, with the adverse effect of gas film removal being stronger for plants in saline than in the non-saline solution. Both previously emergent and submerged plants from the 100 mol m^{-3} NaCl treatment had ~ 25% higher rates of underwater P_N for leaflets than those from the non-saline treatment; this effect might be related to the presence of a more prominent gas film on leaves of plants exposed to 100 mol m^{-3} NaCl than on leaflets from plants in the non-saline treatment (*personal observation*).

The 100 mol m^{-3} NaCl treatment reduced concentrations of chlorophyll *a* and *b* in leaflets from emergent shoots (55% of non-saline controls) and also in leaflets of submerged shoots (71% of non-saline controls; Table 2). When emergent (i.e. shoots in air), the plants brushed with dilute Triton X had lower leaflet chlorophyll in the non-saline conditions, whereas in the saline conditions there

was no effect on plants brushed with dilute Triton X (Table 2). When submerged, removal of gas films did not influence chlorophyll concentrations in leaflets of plants in non-saline conditions, whereas at 100 mol m^{-3} NaCl the leaflet chlorophyll *a* and *b* were 30-40% less when gas films had been removed, compared to leaflets with intact gas films (Table 2).

Leaf gas films – influence on dry mass during submergence

Submerged plants without gas films suffered greater declines in shoot DM than those with gas films intact (Fig. 6). The marked decline in shoot DM after 3 d of submergence (Fig. 6a) coincided with loss (detachment near petiole base) of older leaves. Shoot RGRs of all submerged plants were near zero or negative, reflecting tissue losses, but leaf gas film removal resulted in greater losses of shoot tissues (i.e. more negative RGR) and these losses were also greatest for plants submerged in 100 mol m^{-3} NaCl (Fig. 6b). By contrast to the shoots which lost DM, the root DM of submerged plants in all treatments did not differ to the initial values (data not shown; mean root DM after 6 d of submergence was 0.13 g per plant).

Following de-submergence, the plants submerged in 100 mol m^{-3} NaCl wilted and then desiccated, whereas the plants submerged in non-saline solution initially wilted and then recovered and grew (whole plant RGR data in Supporting Information Table 1S).

There was also no effect of leaf gas film removal (i.e. brushing leaves of emergent plants with 0.1% v/v Triton X, and rinsing) on the growth of plants when the shoots were maintained in air (Supporting Information Table 1S), supporting that the responses of plants to this pre-treatment to prevent gas film retention were not an artefact of the brushing.

Discussion

Leaf gas films delayed salt intrusion into leaves of plants submerged in saline water. This role of leaf gas films in influencing Na^+ and Cl^- ingress and thus plant tolerance of saline submergence, adds to the previously recognised role of these films in enhancement of gas exchange of submerged plants (Raskin & Kende 1983; Colmer & Pedersen 2008b). This function of leaf gas films of diminishing Na^+ and Cl^- entry into submerged leaves of *M. siculus* was of significance to survival of short-term saline submergence.

Gas films occur on superhydrophobic leaves when submerged; a feature now recognised for several terrestrial wetland species that facilitates underwater gas exchange (Pedersen & Colmer 2012). Leaf gas films enhance CO_2 uptake for underwater photosynthesis in light, and O_2 uptake for respiration in darkness; both elevate shoot pO_2 and this enhances internal aeration of submerged tissues (Pedersen *et al.* 2009; Winkel *et al.* 2011). The present study demonstrates that leaf gas films also restrict salt intrusion into leaves during saline submergence. The removal of gas films from leaves of *M. siculus* increased entry of Na^+ and Cl^-, so that tissue concentrations were more than double those in leaves with intact gas films (Fig. 3). The function of leaf gas films in restricting ion uptake would most likely be the result of the thin gas layer preventing direct contact of the saline water with the leaf surface. In addition, the enhanced tissue aeration and photosynthesis resulting from gas films might also aid cellular energy status and thus functioning of ion transporters (c.f. situation for hypoxic roots, Pang *et al.* 2006; Colmer & Greenway 2011). Nevertheless, the physical separation of leaf surface and saline water is likely of most importance since water and ions can be absorbed by leaves (Burkhardt *et al.* 2012; Eller *et al.* 2013).

The role of gas films in hindering ion entry into submerged leaves was evident in the time-series measurements for plants submerged in 100 mol m^{-3} NaCl, which had large increases in tissue Na^+ and Cl^- in submerged leaves on the third day (Fig. 3), coinciding with the disappearance of gas films. The tissue Na^+ and Cl^- concentrations increased further in subsequent days and after 6-7 d of submergence reached presumably toxic levels as *M. siculus* did not survive. Plants submerged in 100 mol m^{-3} NaCl in the dose-response experiment also did not recover upon de-submergence, whereas those previously in 25 or 50 mol m^{-3} NaCl for 7 d resumed growth following de-submergence. The relatively short-term benefits of leaf gas films on *M. siculus* are consistent with the view of Colmer & Voesenek (2009) who classified leaf gas films as an adaptive trait for short-duration submergence.

In addition to the newly identified function described above for leaf gas films in preventing ion intrusion into tissues when under saline submergence, leaf gas films also enhanced underwater gas exchange and internal aeration of submerged *M. siculus*, as found previously for submerged rice (Pedersen *et al.* 2009) and *Spartina anglica* (Winkel *et al.* 2011). Similar to these two other species, the gas films on leaves of *M. siculus* enhanced CO_2 entry for underwater P_N (Fig. 5) and in darkness O_2 entry into leaves and internal diffusion along the petiole (Fig. 4). Interestingly, the

422 measurements of underwater P_N also showed that leaves of plants with roots pre-exposed to salinity
423 had higher rates than leaves from non-saline plants (Fig. 5). This effect of pre-exposure to salinity
424 was visually associated with more prominent gas films on these leaves when submerged. Further
425 studies are needed to determine how any salt-induced structural or chemical alterations of the
426 cuticle might influence leaf hydrophobicity and leaf gas film formation/persistence upon
427 submergence. Differences in leaf hydrophobicity, as dependent on growth conditions, have been
428 described for other species (seasonal changes in hydrophobicity observed in species of e.g., beech,
429 oak and ginkgo; Neinhuis & Barthlott 1998).
430
431 Leaf gas films of *M. siculus* protected against ion intrusion and facilitated gas exchange under
432 saline submergence – but did this contribute to survival and growth of the plants? Plants with gas
433 films did not grow during submergence, even in non-saline water, but when the gas films were
434 removed shoot DM was reduced to half of the initial value (Fig. 6) as older leaves were injured,
435 which then presumably was the cause of these leaves being detached within 3 d of submergence
436 (data not shown). Plants in saline submergence solution suffered the most when leaf gas films were
437 removed, degradation of the shoot was substantial (RGR was $-0.3\ d^{-1}$) presumably due to the high
438 tissue Na^+ and Cl^- concentrations (Fig. 3) having toxic effects. Detrimental effects of high tissue
439 Na^+ and Cl^- concentrations on leaf functioning were evident as a loss of photosynthetic capacity in
440 leaves with gas films removed (Fig. 5). These plants did not survive after de-submergence
441 (Supporting Information Table 1S).
442
443 Although *M. siculus* could only survive complete submergence with low salinity ($\leq$ 50 mol m^{-3}
444 NaCl), this species tolerated waterlogging with high salinity of 500 mol m^{-3} NaCl (Teakle *et al.*
445 2012). When *M. siculus* maintains shoot contact with the atmosphere, the continuum of tissue gas-
446 filled spaces (in petioles, stem/root phellem, and primary aerenchyma in roots) promotes O_2
447 movement into the root system. *M. siculus* with shoots in air can even survive in a severely hypoxic
448 root medium with salinity near that of seawater (Teakle *et al.* 2011). The thick layer of highly
449 porous phellem at the shoot base and extending down the roots (porosity in Supporting Information
450 Table 2S) provides a low-resistance pathway for O_2 transport (i.e. aerenchyma) into and along roots
451 of *M. siculus* (Teakle *et al.* 2011; Verboven *et al.* 2012).
452

In summary, this study has demonstrated that leaf gas films reduce ion ingress into leaves when submerged by saline water (supporting the first hypothesis in the Introduction) – the thin gas layer separates the floodwater from the leaf surface. The leaf gas films were also beneficial for underwater P_N and internal aeration of submerged plants, supporting the second hypothesis and the present findings with *M. siculus* are also consistent with earlier findings on other species (see Introduction and Discussion). Thus, leaf gas films aid survival of plants exposed to short-term saline submergence. This role of leaf gas films should be evaluated also for other species that experience submergence by saline waters e.g., *Phragmites australis* (Adams & Bate 1999) or rice (Gregorio *et al.* 2002), to build on our present findings for *M. siculus*.

Acknowledgements

The authors gratefully acknowledge funding provided by the Centre for Ecohydrology to N.L. Teakle. We thank the UWA Institute of Advanced Studies for hosting O. Pedersen and the A.W. Howard Memorial Trust for supporting the travel of O. Pedersen to Perth. O. Pedersen acknowledges support from the Danish Research Council (grant No. 09-072482). The authors gratefully acknowledge the valuable technical assistance provided by Sonja Jakob.

Tables

Table 1: Summary of experiments and treatments imposed on *Melilotus siculus.*

Treatment Number	Root-zone salinity (mol m^{-3} NaCl)	Root-zone aeration	Submergence treatment[#]	Submergence salinity (mol m^{-3})	Gas film present*
Dose response experiment					
1	0.1	Stagnant	Emergent	n.a.	n.a.
2	25	Stagnant	Emergent	n.a.	n.a.
3	50	Stagnant	Emergent	n.a.	n.a.
4	100	Stagnant	Emergent	n.a.	n.a.
5	0.1	Stagnant	Submerged	0.1	Yes
6	25	Stagnant	Submerged	25	Yes
7	50	Stagnant	Submerged	50	Yes
8	100	Stagnant	Submerged	100	Yes
Leaf gas films – influence on ion intrusion, underwater photosynthesis and internal aeration					
1	0.1	Stagnant	Emergent	n/a	Yes[1]
2	0.1	Stagnant	Emergent	n/a	No[2]
3	100	Stagnant	Emergent	n/a	Yes[1]
4	100	Stagnant	Emergent	n/a	No[2]
5	0.1	Stagnant	Submerged	0.1	Yes
6	0.1	Stagnant	Submerged	0.1	No
7	100	Stagnant	Submerged	100	Yes
8	100	Stagnant	Submerged	100	No
9	0.1	Aerated	Emergent	n.a.	Yes[1]
10	0.1	Aerated	Emergent	n.a.	No[2]
11	100	Aerated	Emergent	n.a.	Yes[1]
12	100	Aerated	Emergent	n.a.	No[2]
Additional growth conditions to establish supporting information					
S1	0.1	Aerated	Emergent	n.a.	No[2]
S2	100	Aerated	Emergent	n.a.	Yes[1]
S3	100	Aerated	Emergent	n.a.	No[2]
S4	0.1	Stagnant	Submerged	100	Yes
S5	0.1	Stagnant	Submerged	100	No

[#]'Emergent' plants had shoots completely in air and 'Submerged' plants were completely submerged by the solution in the tank so that no shoot parts were in contact with the air. *Gas films were removed by brushing with 0.1% v/v Triton X. Plants with gas films intact were brushed with DI water. . [1,2]Emergent plants do not possess a 'gas film', i.e. [1]brushed with DI water and [2]brushed with 0.1% v/v Triton X, and the shoots remained in air.

Table 2. Impact of saline submergence on chlorophyll concentrations in leaves of *Melilotus siculus*. Prior to submergence, all plants had been pre-treated in the root medium with 0.1 or 100 mol m^{-3} NaCl for 7 d with the last 4 d in stagnant deoxygenated nutrient solution (the various root-zone conditions continued during each respective submergence treatment). Gas films were removed by brushing with 0.1% v/v Triton X. Plants with gas films intact were brushed with DI water. Values are the mean (±SE, *n*=4).

Submergence treatment (mol m^{-3} NaCl)	Gas films present	Leaflet Chl_a (µg g^{-1} FM)	Leaflet Chl_b (µg g^{-1} FM)	Leaflet Chl *a:b*
Emergent 0.1	Yes[1]	456 ± 68	103 ± 14	4.4 ± 0.1
Emergent 0.1	No[2]	365 ± 37	81 ± 9.1	4.5 ± 0.1
Emergent 100	Yes[1]	249 ± 21	56 ± 1.6	4.4 ± 0.3
Emergent 100	No[2]	241 ± 23	52 ± 5.2	4.6 ± 0.04
Submerged 0.1	Yes	472 ± 21	119 ± 4.1	4.0 ± 0.06
Submerged 0.1	No	471 ± 20	117 ± 5.5	4.0 ± 0.06
Submerged 100	Yes	338 ± 38	83 ± 9.2	4.1 ± 0.03
Submerged 100	No	196 ± 22	58 ± 6.1	3.4 ± 0.15
$LSD_{0.05}$		*75.6*	*17.6*	*n.s.*

[1,2]Emergent plants do not possess a 'gas film', i.e. [1]brushed with DI water and [2]brushed with 0.1% v/v Triton X, and the shoots remained in air.

Figure legends

Figure 1. Photograph of submerged *Melilotus siculus* plants showing leaf gas films. Plants were submerged with gas films removed (-GF) using 0.1% v/v Triton X (left) or with gas films intact (+GF) (right). *M. siculus* exhibits a hyponastic response (reorientation of petioles towards the vertical direction) when submerged, but it does not display a shoot elongation response.

Figure 2. Shoot ion concentrations of *Melilotus siculus* in response to increasing levels of salinity combined with submergence for 7 d (dose response experiment). Plants were submerged 28 d after imbibition. Prior to submergence, all plants had been pre-treated in the root medium with either 0.1, 25, 50 or 100 mol m^{-3} NaCl for 7 d with the last 4 d in stagnant deoxygenated nutrient solution (the various root-zone conditions continued during each respective submergence treatment). Leaf gas films were present on all plants and were not artificially manipulated in this experiment. Values are the mean (±SE, n=4). Aerated controls for 0.1 and 100 mol m^{-3} NaCl treatments were (µmol g^{-1} DM): Cl^-, 56 ± 4.6 and 872 ± 65; Na^+, 165 ± 4.4 and 1363 ± 53; K^+, 1512 ± 85 and 1072 ± 50 (mean ±SE, n=4).

Figure 3. Effects of gas film removal on concentrations of Cl^-, Na^+ and K^+ in the youngest fully expanded leaves (leaflets only) and whole shoots of *Melilotus siculus*, with time after submergence in saline solution. Plants were submerged 28 d after imbibition for a total of 6 d. Prior to submergence, all plants had been pre-treated in the root medium with 0.1 or 100 mol m^{-3} NaCl for 7 d with the last 4 d in stagnant deoxygenated nutrient solution (the various root-zone conditions continued during each respective submergence treatment). Gas films were removed by brushing with 0.1% v/v Triton X (-GF). Plants with gas films intact were brushed with DI water (+GF). Values are the mean (± SE, n=4).

Figure 4. O_2 dynamics of petioles of *Melilotus siculus* in response to submergence in the dark and the influence of leaf gas films. O_2 microelectrodes were inserted 50 mm below the leaflets, with the petiole in stagnant deoxygenated 0.1% w/v agar submergence medium. Gas films were removed by brushing with 0.1% v/v Triton X (-GF). Plants with gas films intact were brushed with DI water (+GF). Values are the mean ± SE (n=8). Different letters indicate significant differences between treatments (P<0.05) based on Tukey's test.

Figure 5. Underwater net photosynthesis (P_N) of *Melilotus siculus* in response to submergence in non-saline or saline solution and with presence or removal of leaf gas films. Plants were submerged 28 d after imbibition. Prior to submergence, all plants had been pre-treated in the root medium with 0.1 or 100 mol m^{-3} NaCl for 7 d with the last 4 d in stagnant deoxygenated nutrient solution (the various root-zone conditions continued during each respective submergence treatment). Gas films were removed by brushing with 0.1% v/v Triton X (-GF). Plants with gas films intact were brushed with DI water (+GF). Leaflets were removed 3 d after submergence treatments commenced and underwater P_N measured for samples from plants that had been with shoots in air or submerged (with or without gas films). Values are the mean (±SE, n=4). Different letters indicate significant differences between treatments (P<0.05) based on Tukey's test. Aerial photosynthesis was measured on intact emergent plants (i.e. shoots in air) and the average value for young fully expanded leaves in air was 20.6 ± 1.7 µmol CO_2 m^{-2} s^{-1}.

Figure 6. Shoot dry mass (DM, a) and shoot relative growth rate (RGR, b) of *Melilotus siculus* in response to submergence with gas films intact (+GF) or removed (-GF) in saline and non-saline solutions for 6 d. Plants were submerged 28 d after imbibition. Prior to submergence, all plants had been pre-treated in the root medium with 0.1 or 100 mol m^{-3} NaCl for 7 d with the last 4 d in stagnant deoxygenated nutrient solution (the various root-zone conditions continued during each respective submergence treatment). Gas films were removed by brushing with 0.1% v/v Triton X (-GF). Plants with gas films intact were brushed with DI water (+GF). Values are the mean (±SE, n=4). Different letters represent a significant difference between treatments (P<0.05) based on Dunn's post hoc test. Values for root DM did not change during the treatment period and were not significantly different between treatments (average 0.127 g). DM for the emergent plants (i.e. with shoots in air) at day 6 were (g $plant^{-1}$): 0.1 mol m^{-3} NaCl, 0.71 ± 0.03 (shoot) and 0.28 ± 0.02 (root); 100 mol m^{-3} NaCl, 0.45 ± 0.04 (shoot) and 0.18 ± 0.01 (root).

References

Adams J.B. & Bate G.C. (1999) Growth and photosynthetic performance of *Phragmites australis* in estuarine waters: a field and experimental evaluation. *Aquatic Botany* **64**, 359-367.

Armstrong W. (1979) Aeration in higher plants. *Advances in Botanical Research* **7**, 225-332.

Bailey-Serres J. & Voesenek L.A.C.J. (2008) Flooding stress: Acclimations and genetic diversity. *Annual Review of Plant Biology* **59**, 313-339.

Barrett-Lennard E.G. (2003) The interaction between waterlogging and salinity in higher plants: causes, consequences and implications. *Plant and Soil* **253**, 35-54.

Barrett-Lennard E.G. & Shabala S.N. (2013) The waterlogging/salinity interaction in higher plants revisited–focusing on the hypoxia-induced disturbance to K^+ homeostasis. *Functional Plant Biology* **40**, 872-882.

Bonython A.L., Ballard R.A., Charman N., Nichols P.G.H. & Craig A.D. (2011) New strains of rhizobia that nodulate regenerating messina (*Melilotus siculus*) plants in saline soils. *Crop and Pasture Science* **62**, 427-436.

Burkhardt J., Basi S., Pariyar S. & Hunsche M. (2012) Stomatal penetration by aqueous solutions - an update involving leaf surface particles. *New Phytologist* **196**, 774-787.

Colmer T.D. & Flowers T.J. (2008) Flooding tolerance in halophytes. *New Phytologist* **179**, 964-974.

Colmer T.D. & Greenway H. (2011) Ion transport in seminal and adventitious roots of cereals during O_2 deficiency. *Journal of Experimental Botany* **62**, 39-57.

Colmer T.D. & Pedersen O. (2008a) Oxygen dynamics in submerged rice (*Oryza sativa*). *New Phytologist* **178**, 326-334.

Colmer T.D. & Pedersen O. (2008b) Underwater photosynthesis and respiration in leaves of submerged wetland plants: gas films improve CO_2 and O_2 exchange. *New Phytologist* **177**, 918-926.

Colmer T.D. & Voesenek L.A.C.J. (2009) Flooding tolerance: suites of plant traits in variable environments. *Functional Plant Biology* **36**, 665-681.

Colmer T.D., Winkel A. & Pedersen O. (2011) A perspective on underwater photosynthesis in submerged terrestrial wetland plants. *AoB PLANTS* **2011**, plr030.

Eller C.B., Lima A.L. & Oliveira R.S. (2013) Foliar uptake of fog water and transport belowground alleviates drought effects in the cloud forest tree species, *Drimys brasiliensis* (Winteraceae). *New Phytologist*,

Flowers T.J. & Colmer T.D. (2008) Salinity tolerance in halophytes. *New Phytologist* **179**, 945-963.

Greenway H. & Gibbs J. (2003) Mechanisms of anoxia tolerance in plants. II. Energy requirements for maintenance and energy distribution to essential processes. *Functional Plant Biology* **30**, 999-1036.

Gregorio G.B., Senadhira D., Mendoza R.D., Manigbas N.L., Roxas J.P. & Guerta C.Q. (2002) Progress in breeding for salinity tolerance and associated abiotic stresses in rice. *Field Crops Research* **76**, 91-101.

Jackson M.B. & Drew M.C. (1984) Effects of flooding on growth and metabolism of herbaceous plants. In: *Flooding and plant growth* (ed T.T. Kozlowski), pp. 47-128. Academic Press, New York.

Lang A.R.G. (1967) Osmotic coefficients and water potentials of sodium chloride solutions from 0 to 40^{o}C. *Australian Journal of Chemistry* **20**, 2017-2023.

Marañòn T., Romero J.M. & Murillo J.M. (1989) *Salt tolerant legumes from the Guadalquivir delta (S.W. Spain)*. Paper presented at the XVI International Grassland Congress, Nice, France.

589 Munns R. & Tester M. (2008) Mechanisms of salinity tolerance. *Annual Review of Plant Biology*
590 **59**, 651-681.
591 Munns R., Wallace P.A., Teakle N.L. & Colmer T.D. (2010) Measuring soluble ion concentrations
592 (Na^+, K^+, Cl^-) in salt-treated plants In: *Plant Stress Tolerance: Methods and Protocols* (ed
593 R. Sunkar), pp. 371-382. Springer, Heidelberg.
594 Neinhuis C. & Barthlott W. (1997) Characterization and distribution of water-repelent, self-cleaning
595 plant surfaces. *Annals of Botany* **79**, 667-677.
596 Neinhuis C. & Barthlott W. (1998) Seasonal changes of leaf surface contamination in beech, oak,
597 and ginkgo in relation to leaf micromorphology and wettability. *New Phytologist* **138**, 91-
598 98.
599 Pang J.Y., Newman I., Mendham N., Zhou M. & Shabala S. (2006) Microelectrode ion and O_2
600 fluxes measurements reveal differential sensitivity of barley root tissues to hypoxia. *Plant,*
601 *Cell & Environment* **29**, 1107-1121.
602 Pedersen O. & Colmer T.D. (2012) Physical gills prevent drowning of many wetland insects,
603 spiders and plants. *Journal of Experimental Biology* **215**, 705-709.
604 Pedersen O., Colmer T.D. & Sand-Jensen K. (2013) Underwater photosynthesis of submerged
605 plants – recent advances and methods. *Frontiers in Plant Science* **4**,
606 Pedersen O., Rich S.M. & Colmer T.D. (2009) Surviving floods: leaf gas films improve O_2 and CO_2
607 exchange, root aeration, and growth of completely submerged rice. *The Plant Journal* **58**,
608 147-156.
609 Pedersen O., Rich S.M., Pulido C., Cawthray G.R. & Colmer T.D. (2011) Crassulacean acid
610 metabolism enhances underwater photosynthesis and diminishes photorespiration in the
611 aquatic plant *Isoetes australis*. *New Phytologist* **190**, 332-339.
612 Plett C.D. & Møller I.S. (2010) Na^+ transport in glycophytic plants: what we know and would like
613 to know. *Plant, Cell & Environment* **33**, 612-626.
614 Raskin I. (1983) A method for measuring leaf density, thickness and internal gas. *Hortscience* **18**,
615 698-699.
616 Raskin I. & Kende H. (1983) How does deep water rice solve its aeration problem? *Plant*
617 *Physiology* **72**, 447-454.
618 Rogers M.E., Colmer T.D., Frost K., Henry D., Cornwall D., Hulm E., Deretic J., Hughes S.R. &
619 Craig A.D. (2008) Diversity in the genus *Melilotus* for tolerance to salinity and
620 waterlogging. *Plant and Soil* **304**, 89-101.
621 Setter T.L., Waters I., Wallace I., Bekhasut P. & Greenway H. (1989) Submergence of rice. I.
622 Growth and photosynthetic response to CO_2 enrichment of floodwater. *Australian Journal*
623 *of Plant Physiology* **16**, 251-263.
624 Shabala S.N. & Mackay A. (2011) Ion transport in halophytes. In: *Advances in Botanical Research*
625 (eds J.C. Kader & M. Delseny), pp. 151-199. Academic Press, Burlington.
626 Teakle N.L., Armstrong J., Barrett-Lennard E.G. & Colmer T.D. (2011) Aerenchymatous phellem
627 in hypocotyl and roots enables O_2 transport in *Melilotus siculus*. *New Phytologist* **190**, 340-
628 350.
629 Teakle N.L., Bowman S., Barrett-Lennard E.G., Real D. & Colmer T.D. (2012) Comparisons of
630 annual pasture legumes in growth, ion regulation and root porosity demonstrate that
631 *Melilotus siculus* has exceptional tolerance to combinations of salinity and waterlogging.
632 *Environmental and Experimental Botany* **77**, 175-184.
633 Teakle N.L. & Tyerman S.D. (2010) Mechanisms of Cl^- transport contributing to salt tolerance.
634 *Plant, Cell & Environment* **33**, 566-589.

635 Thomson C.J., Armstrong W., Waters I. & Greenway H. (1990) Aerenchyma formation and
636 associated oxygen movement in seminal and nodal roots of wheat. *Plant, Cell &*
637 *Environment* **13**, 395-403.
638 Verboven P., Pedersen O., Herremans E., Ho Q.T., Nicolaï B.M., Colmer T.D. & Teakle N. (2012)
639 Root aeration via aerenchymatous phellem: three-dimensional micro-imaging and radial O_2
640 profiles in *Melilotus siculus*. *New Phytologist* **193**, 420-431.
641 Voesenek L.A.C.J., Colmer T.D., Pierik R., Millenaar F.F. & Peeters A.J.M. (2006) How plants
642 cope with complete submergence. *New Phytologist* **170**, 213-226.
643 Wellburn A.R. (1994) The spectral determination of chlorophylls a and b, as well as total
644 carotenoids, using various solvents with spectrophotometers of different resolution. *Journal*
645 *of Plant Physiology* **144**, 307-313.
646 Wiengweera A., Greenway H. & Thomson C.J. (1997) The use of agar nutrient solution to simulate
647 lack of convection in waterlogged soils. *Annals of Botany* **80**, 115-123.
648 Winkel A., Colmer T.D., Ismail A.M. & Pedersen O. (2013) Internal aeration of paddy field rice
649 (*Oryza sativa* L.) during complete submergence – importance of light and floodwater O_2.
650 *New Phytologist* **197**, 1193-1203.
651 Winkel A., Colmer T.D. & Pedersen O. (2011) Leaf gas films of *Spartina anglica* enhance rhizome
652 and root oxygen during tidal submergence. *Plant, Cell & Environment* **34**, 2083-2092.

653

654

Supporting Information Table 1S. Whole plant relative growth rate (RGR; assuming logarithmic growth or decay of tissue) of *Melilotus siculus* during a 6 d submergence period (indicated in table as 0-6 d) and following de-submergence for 7 d (indicated in table as 6-13 d). Plants were submerged 28 d after imbibition. Prior to submergence, all plants had been pre-treated in the root medium with 0.1 or 100 mol m^{-3} NaCl for 7 d with the last 4 d in stagnant deoxygenated nutrient solution (the various root-zone conditions continued during each respective submergence treatment). Gas films were removed by brushing with Triton X. Plants with gas films intact were brushed with DI water.l Values are the mean (±SE, *n*=4).

Submergence treatment (mol m^{-3} NaCl)	Gas films present	RGR g g^{-1} d^{-1} 0-6 d	RGR g g^{-1} d^{-1} 6-13 d
Emergent 0.1	Yes[1]	0.396 ± 0.011[de]	0.157 ±0.038[d]
Emergent 0.1	No[2]	0.364 ± 0.022[d]	0.149 ±0.022[d]
Emergent 100	Yes[1]	0.244 ± 0.044[d]	*n.d.*
Emergent 100	No[2]	0.228 ± 0.025[d]	*n.d.*
Submerged 0.1	Yes	0.031 ± 0.009[c]	0.373 ± 0.085[d]
Submerged 0.1	No	-0.129 ± 0.038[ab]	0.282 ± 0.029[d]
Submerged 100	Yes	-0.026 ± 0.020[b]	*
Submerged 100	No	-0.194 ± 0.049[a]	*

Values with different letters were significantly different (P<0.05, Tukey test)

*indicates plants for this treatment were all dead; *n.d.* = not determined

[1,2]Emergent plants do not possess a 'gas film', i.e. [1]brushed with DI water and [2]brushed with 0.1% v/v Triton X.

Supporting Information Table 2S. Summary of the response of *Melilotus siculus* to complete submergence combined with salinity. Plants were submerged 28 d after imbibition. Prior to submergence, all plants had been pre-treated in the root medium with either 0.1 or 100 mol m^{-3} NaCl for 7 d with the last 4 d in stagnant deoxygenated nutrient solution (the various root-zone conditions continued during each respective submergence treatment). Values are the mean (±SE, *n*=4). See Table 1 for treatment definitions. A: Porosity and shoot mass were measured 6 d after submergence. Plants were then de-submerged and grown with shoots in air for another 7 d to assess recovery. B: Shoot ion concentrations were measured after 6 d submergence.

A.

Submergence treatment	Salinity (mol m^{-3})	Petiole porosity (%)	Tap root porosity (%)	Shoot DM 6 d submerged (g/plant)	Shoot DM 7 d recovery (g/plant)
Emergent	0	12.3 ± 1.1	31.5 ± 2.2	0.48 ± 0.07	0.92 ± 0.2
Submerged	0	4.5 ± 1.1	16.3 ± 4.3	0.078 ± 0.005	0.10 ± 0.04
Emergent	100	6.7 ± 0.7	28.9 ±1.06	0.45 ± 0.01	1.06 ± 0.1
Submerged	100	2.0 ± 0.4	7.9 ± 1.1	0.11 ± 0.01	0.19 ± 0.1
*P-value sub x salt**		*0.369*	*0.038*	*0.405*	*0.361*
$LSD_{0.05}$		*2.2*	*3.2*	*0.05*	*0.12*

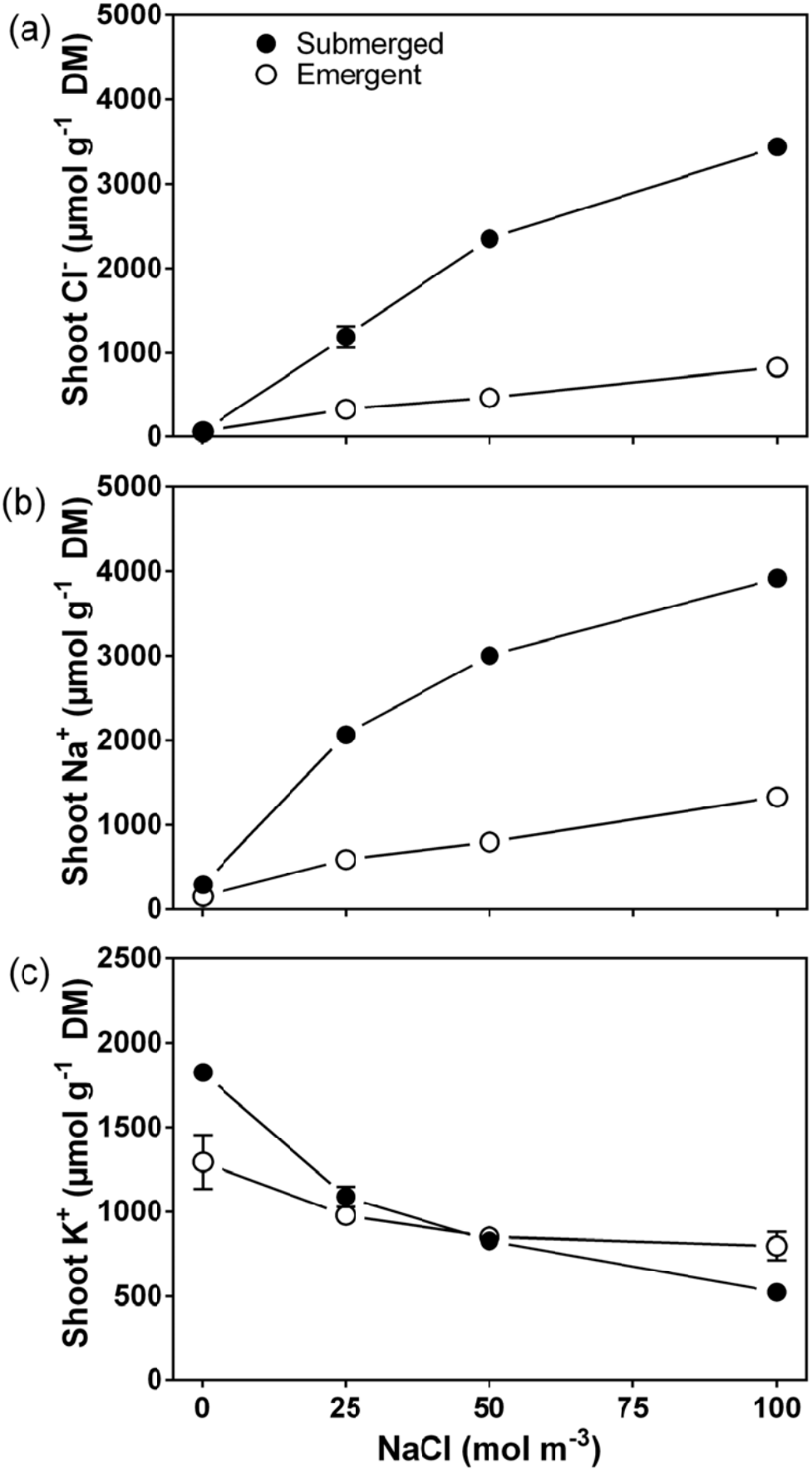
B.

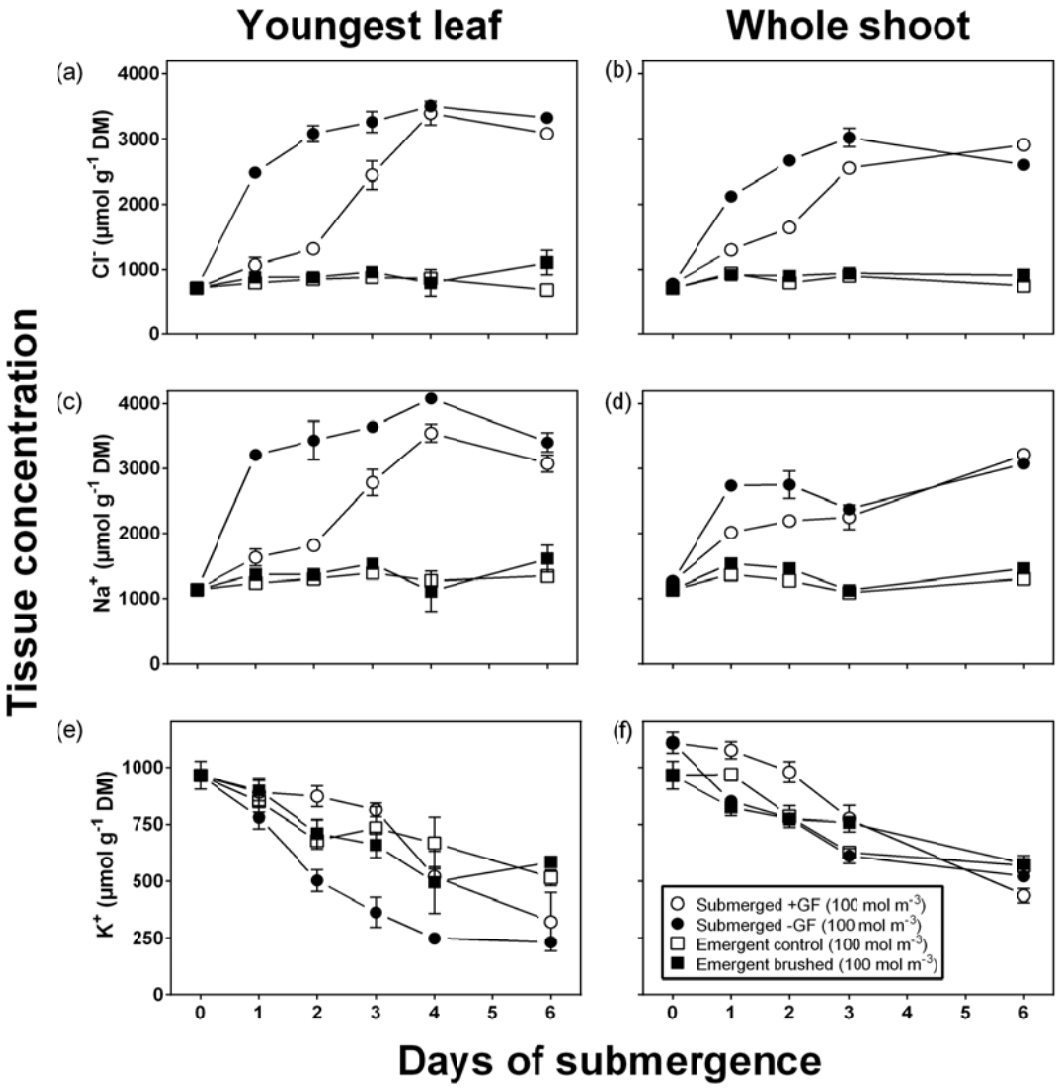
Submergence treatment	Salinity (mol m^{-3})	Shoot concentration (µmol g^{-1} DM)		
		Cl^-	Na^+	K^+
Emergent	0	57 ± 4	148 ± 1	1293 ± 160
Submerged	0	102 ± 6	288 ±17	1824 ± 41
Emergent	100	826 ± 38	1331 ± 76	795 ± 84
Submerged	100	3440 ± 37	3918 ± 69	523 ± 14
*P-value sub x salt**		*<0.001*	*<0.001*	*<0.001*
$LSD_{0.05}$		*52*	*51*	*110*

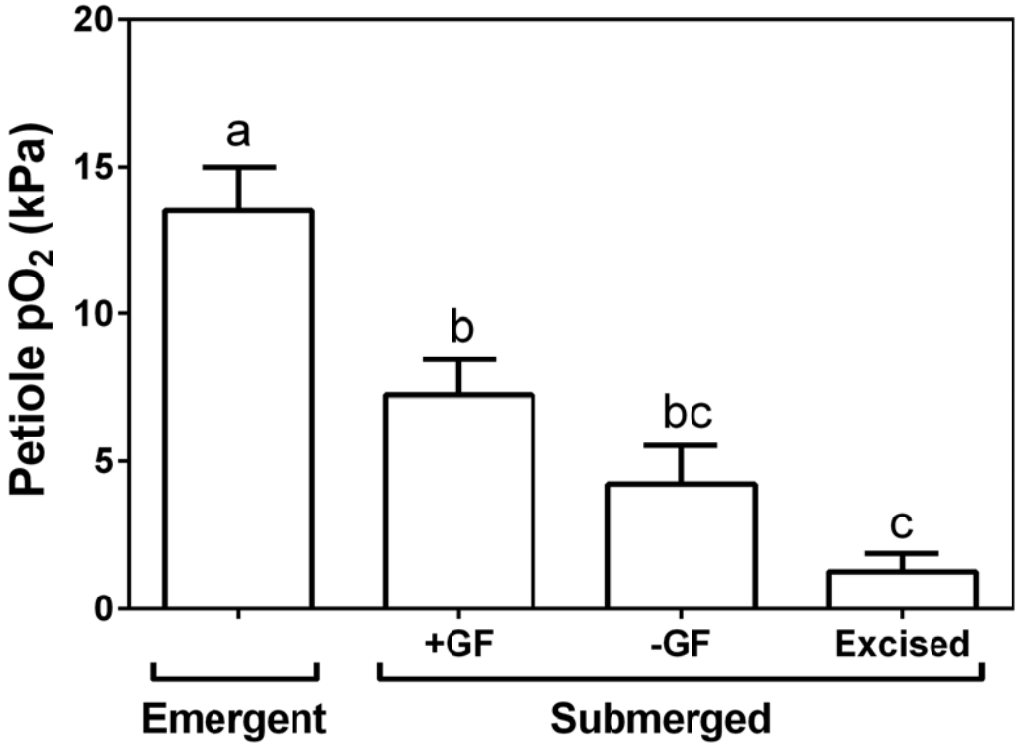
*P-values are given for the interaction between submergence and salinity treatments based on a 2-way ANOVA.

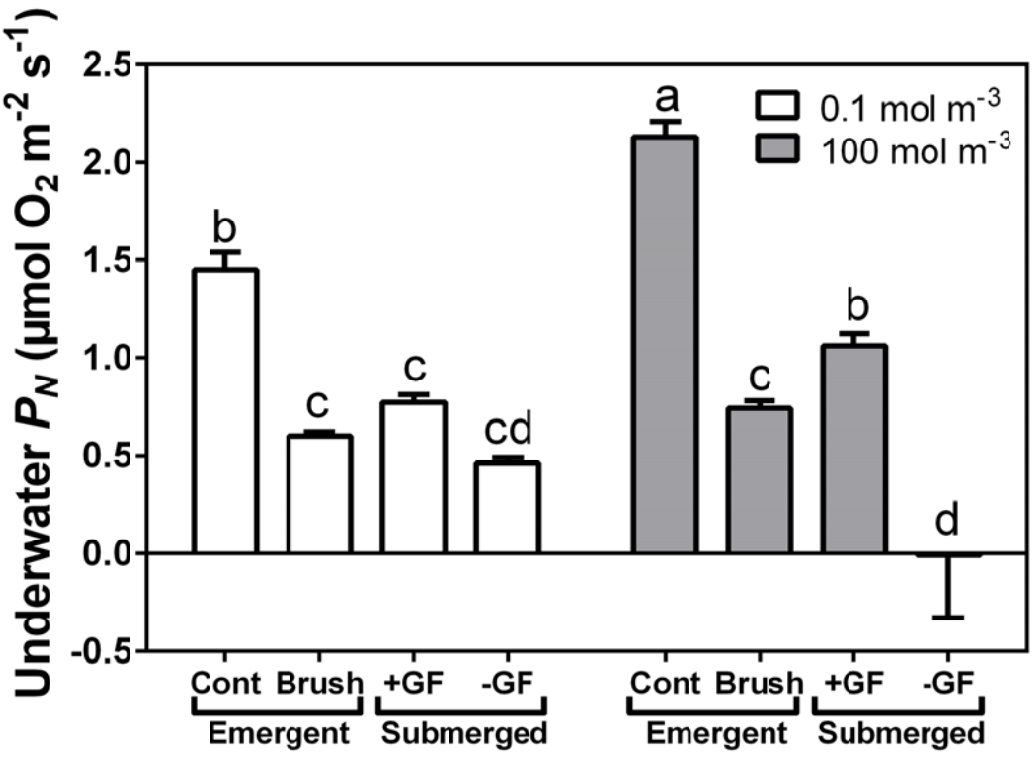
-GF

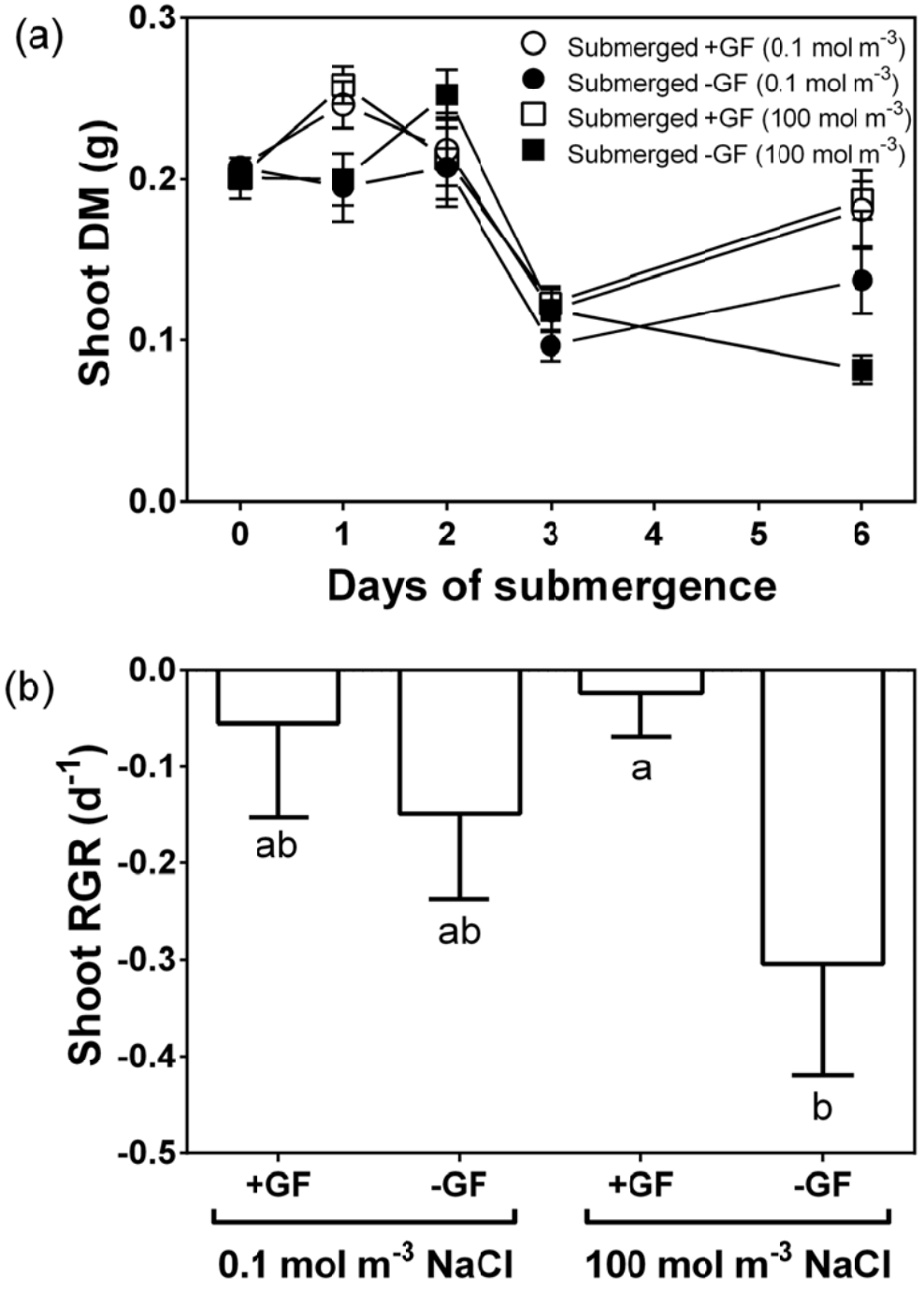
+GF


(a)
Submerged
Emergent
Shoot Cl- (µmol g-1 DM)
5000
4000
3000
2000
1000
0
(b)
Shoot Na+ (µmol g-1 DM)
5000
4000
3000
2000
1000
0
(c)
Shoot K+ (µmol g-1 DM)
2500
2000
1500
1000
500
0
0
25
50
75
100
NaCl (mol m-3)


Youngest leaf
Whole shoot
Tissue concentration
(a)
(b)
(c)
(d)
(e)
(f)
Cl⁻ (µmol g⁻¹ DM)
Na⁺ (µmol g⁻¹ DM)
K⁺ (µmol g⁻¹ DM)
0
1000
2000
3000
4000
250
500
750
1000
0
1
2
3
4
5
6
Days of submergence
Submerged +GF (100 mol m⁻³)
Submerged -GF (100 mol m⁻³)
Emergent control (100 mol m⁻³)
Emergent brushed (100 mol m⁻³)


20
15
10
5
0
Petiole pO2 (kPa)
a
b
bc
c
+GF
-GF
Excised
Emergent
Submerged


Underwater P_N (µmol O_2 m^{-2} s^{-1})
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
0.1 mol m^{-3}
100 mol m^{-3}
b
c
c
cd
a
c
b
d
Cont
Brush
+GF
-GF
Emergent
Submerged
Cont
Brush
+GF
-GF
Emergent
Submerged


(a)
Shoot DM (g)
0.0
0.1
0.2
0.3
0
1
2
3
4
5
6
Days of submergence
Submerged +GF (0.1 mol m-3)
Submerged -GF (0.1 mol m-3)
Submerged +GF (100 mol m-3)
Submerged -GF (100 mol m-3)
(b)
Shoot RGR (d-1)
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
ab
ab
a
b
+GF
-GF
+GF
-GF
0.1 mol m-3 NaCl
100 mol m-3 NaCl