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

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2 *Melilotus siculus* submerged in saline water

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21 **Abstract**

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

35

36 **Keywords**

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

39 **Introduction**

40 Flooding can be a severe abiotic stress on plants (Bailey-Serres & Voesenek 2008) and in various
41 situations the water can be saline. Flooded soils are typically low in O₂ and when shoots are
42 submerged plants face further restrictions on their gas exchange (Armstrong 1979; Voesenek *et al.*
43 2006). Traits associated with plant flooding tolerance include aerenchyma for internal aeration
44 (Armstrong 1979), adventitious roots (Jackson & Drew 1984), anoxia tolerance in some tissues
45 (Greenway & Gibbs 2003), shoot elongation response (Bailey-Serres & Voesenek 2008), and
46 capacity for underwater photosynthesis (Colmer *et al.* 2011). Flooding regimes of different depths
47 and durations, however, impose selection pressures for various combinations of these and other
48 traits in wetland plants (Colmer & Voesenek 2009).

49
50 Salt tolerance is generally associated with the ability to regulate Na⁺, K⁺ and Cl⁻ transport to the
51 shoots (Plett & Møller 2010; Teakle & Tyerman 2010; Shabala & Mackay 2011) and effective ion
52 compartmentation and maintenance of favourable water relations (reviewed by Flowers & Colmer
53 2008; Munns & Tester 2008). When combined with salinity, the low O₂ associated with flooding
54 impacts on the energetic demands of regulating ion transport to prevent shoot Na⁺ and Cl⁻
55 accumulating to toxic levels (Barrett-Lennard 2003; Colmer & Flowers 2008) and to maintain
56 sufficient K⁺ (Barrett-Lennard & Shabala 2013). Surprisingly, very few studies have evaluated the
57 mechanisms of plant tolerance to saline submergence (e.g., in halophytes reviewed by Colmer &
58 Flowers 2008).

59
60 *Melilotus siculus* (Turra) B.D. Jacks. (syn. *Melilotus messanensis*) is a waterlogging- and salt-
61 tolerant annual legume species (Marañón *et al.* 1989; Rogers *et al.* 2008; Teakle *et al.* 2012) used
62 for pasture on some soils/small areas in some regions with Mediterranean climates. Waterlogging
63 tolerance of *M. siculus* is linked to a high capacity for internal root aeration via aerenchymatous
64 phellem (up to 50% porosity, Verboven *et al.* 2012) and formation of numerous new lateral roots of
65 high porosity (Teakle *et al.* 2011). This internal supply of O₂ for respiration provides energy to
66 regulate root ion transport under combined waterlogging and salinity (Teakle *et al.* 2012). Areas
67 naturally inhabited by *M. siculus* (Marañón *et al.* 1989) or saline agricultural land being targeted for
68 pasture production (Bonython *et al.* 2011) can also experience short-term flooding, resulting in
69 plant submergence. Tolerance of *M. siculus* to submergence has not been previously studied,
70 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 reputedly 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 CaSO₄ 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 KH₂PO₄, 3.0 KNO₃, 4.0 Ca(NO₃)₂, 1.0 MgSO₄; and micronutrients (mmol m⁻³): 37.5

103 FeNa₃EDTA, 23.0 H₃BO₃, 4.5 MnCl₂, 4.0 ZnSO₄, 1.5 CuSO₄, and 0.05 MoO₃, as used previously
104 for this species (e.g., Rogers *et al.* 2008; Teakle *et al.* 2011). NaCl concentration was 0.1 mol m⁻³.
105 Solution pH was buffered with 2.5 mol m⁻³ MES (2-[N-Morpholino]ethanesulfonic acid) adjusted
106 with KOH to pH 6.3. Nutrient solutions were aerated, changed weekly and topped up with DI water
107 as required. All pots were covered with Al-foil to exclude light. Plants were kept for the duration of
108 the experiment in a naturally lit, temperature controlled (20/15°C day/night) phytotron during
109 September to October 2010 in Perth, Western Australia. Average photosynthetically active radiation
110 (PAR) within the phytotron at midday during the experimental period was 1149 μmol m⁻² s⁻¹.
111

112 *Root-zone salinity and O₂ treatments*

113 Salinity treatments in the root-zone medium were imposed 21 d after imbibition, by adding 25 mol
114 m⁻³ NaCl increments every 12 h to reach the final concentrations of 25, 50 or 100 mol m⁻³, also with
115 control solutions maintained at 0.1 mol m⁻³ NaCl. Two days after the final NaCl concentrations
116 were reached, a hypoxic pre-treatment was given to the root-zone medium of stagnant designated
117 pots by bubbling with N₂ until the O₂ level was approximately 10% of that at air-equilibrium. After
118 24 h, the solutions in these pots were changed to a deoxygenated stagnant 0.1% (w/v) agar nutrient
119 solution (Wiengweera *et al.* 1997), with the mineral composition as described above. Plants were
120 then grown for an additional 3 d in this stagnant root-zone medium prior to imposition of the shoot
121 submergence treatments.
122

123 *Submergence treatment*

124 For the submergence treatments, individual plants were carefully removed from the 4.5 l pots and
125 the intact roots placed (with the same foam holder around the stem base) into a 250 ml black plastic
126 bottle containing the same nutrient solution (with the same salinity level and also containing de-
127 oxygenated stagnant agar solution) as for each particular plant in the various root-zone treatments
128 described in the preceding section. Each bottle also contained 7 glass marbles to weigh the bottle
129 down in the submergence tanks. The top of the bottle and foam were wrapped in parafilm, to
130 impede the possibility of nutrients moving through the foam holder, which was acting like a plug in
131 the bottle neck. The bottles containing one plant each were then transferred into clear Perspex
132 cylinders filled with 12 l of solution (shoots completely submerged and unable to reach air). The
133 submergence solution contained 2 mol m⁻³ CaSO₄, 0.25 mol m⁻³ MgSO₄ and 1 mol m⁻³ KHCO₃.
134 Dissolved CO₂ was maintained at 140 mmol m⁻³ (with pH at 7.2) using a pressurised CO₂ cylinder

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

141

142 *Response to submergence with different salinity levels*

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

151

152 *Role of leaf gas films in tolerance to saline submergence*

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

160

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

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

169

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

175

176 *Harvests and fresh and dry mass measurements*

177 An initial harvest for root and shoot fresh mass (FM) and DM was taken 21 d after imbibition
178 (when NaCl treatment commenced). Additional harvests were then taken at the time of
179 submergence (28 d after imbibition, after the salt and stagnant root-zone pre-treatments) and at
180 various subsequent intervals for the different experiments (see above and figure legends). Young,
181 fully expanded leaves were sampled *in situ* for measurements of underwater net photosynthesis and
182 tissue chlorophyll concentration (details below). Plants were then removed from the tanks and
183 bottles, and roots were washed 3 times (for 10 s each time) in 4 mol m⁻³ CaSO₄ with mannitol at a
184 concentration iso-osmotic to the root-zone NaCl treatment (Lang 1967). Roots were separated from
185 shoots and maximum root length and FM were recorded. Shoots were rinsed in 2 mol m⁻³ CaSO₄
186 with mannitol at a concentration iso-osmotic to the submergence NaCl treatment and tissues
187 collected for measurements of mass and ion concentrations. Total shoot FM was recorded and
188 tissues were oven dried at 60°C for 3 d, after which DM was recorded and tissue ion concentrations
189 were measured (see next section). Extra plants were used for measuring petiole and tap root
190 porosity (described below).

191

192 *Concentrations of tissue ions*

193 Concentrations of Na⁺, K⁺ and Cl⁻ were measured in dried samples of leaflets or whole shoots. 100
194 mg of finely ground dried tissue was extracted in 10 ml of 0.5 M HNO₃ for 2 d at 30 °C (Munns *et*
195 *al.* 2010). Extracts were measured for Na⁺ and K⁺ (Jenway PFP7 Flame Photometer, Essex UK) and
196 Cl⁻ (Slamed Chloridometer CHL 50, Frankfurt Germany). The reliability of these analyses was
197 confirmed by taking a reference plant sample (ASPAC #85) with known ionic composition through
198 the same procedures.

199

200 *Underwater net photosynthesis*

201 The youngest, fully expanded leaves were removed from plants that had been submerged for 3 d.
202 Underwater net photosynthesis (P_N) was measured essentially as described by Pedersen *et al.*
203 (2013). Gas films were removed from half of the samples by brushing with 0.1% v/v Triton X and
204 then rinsing using a solution of the same composition as the incubation medium. Leaves were
205 carefully added to 25 mL glass vials containing incubation solution, which was the same as the
206 submergence solution the plants had come from (i.e. with either 0.1 or 100 mol m⁻³ NaCl in the
207 basal medium of 2.0 mol m⁻³ CaSO₄, 0.25 mol m⁻³ MgSO₄ and 1.0 mol m⁻³ KHCO₃) plus 5.0 mol
208 m⁻³ TES (2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid) buffer to
209 maintain the pH at 7.2 after adjustment with 0.5 M HCl, so that dissolved CO₂ was at 140 mmol
210 m⁻³. The pO₂ of the solution was adjusted to approximately 10 kPa by mixing equal volumes of N₂
211 or air-bubbled solution; starting with O₂ below air-equilibrium helps to prevent photorespiration
212 (Setter *et al.* 1989; Pedersen *et al.* 2011). The top of the glass vials were sealed with Parafilm® and
213 aluminium foil, instead of glass stoppers, to prevent the pressure that can occur from stopper
214 insertion causing the gas film to ‘collapse’ on these leaves. Blank vials without leaves were also
215 included. Vials were incubated on a rotating wheel within an illuminated (PAR = 700 μmol m⁻² s⁻¹)
216 water bath (20°C) for 60 min. Dissolved O₂ concentrations were then measured using a Clarke-type
217 O₂ microelectrode (OX-25, Unisense A/S, Aarhus, Denmark). Immediately after these
218 measurements, leaflet samples were weighed and leaflet area measured using a leaf area meter (Li-
219 Cor LI-3000, Lincoln, USA).

220

221 Net photosynthesis for leaves of intact plants in air was also measured. An infra-red gas analyser
222 (Li-Cor LI-6400) attached to a leaf cuvette was used to measure light-saturated net photosynthesis
223 by young, fully expanded leaves at ambient CO₂ (390 μmol mol⁻¹) with PAR of 1500 μmol m⁻² s⁻¹
224 (maximum PAR in phytotron).

225

226 *Porosity measurements*

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

231 0.2 g FM of petiole tissue was used. Approximately 0.5 g FM of the upper part of the tap root
232 containing phellem (Teakle *et al.* 2011) was used for root samples.

233

234 *Chlorophyll analysis*

235 Leaflet samples from plants that had been submerged for 3 d were frozen in liquid N₂ and freeze-
236 dried. 20 mg of ground sample was extracted in 1.25 ml of cold 100% methanol for 30 min in
237 darkness (Wellburn 1994). Samples were centrifuged for 10 min at 1000 rpm in a microcentrifuge
238 at 4°C. The supernatant was removed and 2 µl analysed across 220 to 750 nm using a NanoDrop
239 Spectrophotometer (ND 1000, Thermo Scientific, Asheville, USA). Based on the nanodrop
240 resolution of 3 nm, the following equations from Wellburn (1994) were used to calculate
241 chlorophylls *a* (Chl *a*) and *b* (Chl *b*) using absorbance (Abs) at 653 and 666 nm.

242 *Equation 1:* $\text{Chl } a = 15.65 \times \text{Abs}_{666} - 7.34 \times \text{Abs}_{653}$

243 *Equation 2:* $\text{Chl } b = 27.05 \times \text{Abs}_{653} - 11.21 \times \text{Abs}_{666}$

244

245 *Internal pO₂ of petioles*

246 Internal pO₂ at the distal end of petioles of excised leaves was measured when reliant on internal O₂
247 movement, to evaluate O₂ entry and supply via leaflets when in air (with the petiole in
248 deoxygenated medium) or when submerged with or without leaf gas films. Each leaf with petiole
249 (approximately 100 mm in length) was mounted on a stainless steel mesh in a trough following the
250 procedure of Colmer & Pedersen (2008a). The petiole was immersed in 0.1% w/v deoxygenated
251 agar prepared in the same submergence solution as above, whereas the leaf with its 3 leaflets
252 protruded out of the trough (i.e. leaflets exposed to air). An O₂ microelectrode (tip diameter 25 µm,
253 OX25, Unisense A/S, Denmark) connected to a pA meter (Multimeter, Unisense A/S, Denmark)
254 was inserted 150 µm into the petiole, 50 mm below the leaflets, and pO₂ was followed over time.
255 Petiole pO₂ was measured *i*) with the leaflets exposed to air, *ii*) with the leaflets submerged with
256 intact gas films, *iii*) with the leaflets submerged but with the gas films removed (see above), and *iv*)
257 with the leaflets severed. Measurements were taken at 20 °C in darkness.

258

259 *Data analyses*

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

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

270

271 **Results**

272 *Response to submergence with different salinity levels*

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

277

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

282

283 As hypothesised, shoot Na^+ and Cl^- concentrations increased with each higher NaCl treatment; the
284 increases in concentrations of these ions in the shoots of submerged plants were far greater than
285 those of emergent plants (Fig. 2). A dose-effect was also evident under both flooding conditions.
286 For example, shoot Cl^- concentrations of emergent as well as completely submerged plants were
287 2.3-fold higher in plants exposed to high salt (100 mol m^{-3} NaCl) compared to those in low salt (25
288 mol m^{-3} NaCl) concentration. Shoot Na^+ concentration also showed a similar dose-dependent
289 pattern for emergent and submerged plants, but again with higher overall concentrations in the
290 submerged plants compared to plants with emergent shoots. Salt exposure reduced shoot K^+
291 concentrations in both emergent and submerged plants; shoot K^+ did not differ between emergent
292 and submerged plants in 25 and 50 mol m^{-3} NaCl, but it was 34% less for submerged plants at 100
293 mol m^{-3} NaCl (Fig. 2c).

294

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

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

328

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

330 O_2 dynamics were evaluated in petioles when reliant on O_2 diffusion via the lamina (i.e. leaflet)
331 surfaces. Leaves with petioles of ~ 100 mm length were excised and then the petiole portion was
332 submerged in deoxygenated 0.1% agar submergence solution, all in darkness, so that the only
333 source of O_2 would be via longitudinal internal diffusion. A microelectrode measured tissue O_2 at
334 the distal end, initially with the lamina in air and then following lamina submergence, removal of
335 leaf gas films, and finally lamina excision. As expected (see Introduction), O_2 status of the petiole
336 declined upon submergence of the leaflets in water at air-equilibrium, and removal of gas films
337 further restricted the supply of O_2 to the petiole (Fig. 4). Excision of the leaflets caused $p\text{O}_2$ to drop
338 within the petiole to under 2 kPa (Fig. 4); then, entry of O_2 would have been only via the short stub
339 of petiole and cut surfaces remaining in the water.

340

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

353

354 The 100 mol m^{-3} NaCl treatment reduced concentrations of chlorophyll *a* and *b* in leaflets from
355 emergent shoots (55% of non-saline controls) and also in leaflets of submerged shoots (71% of non-
356 saline controls; Table 2). When emergent (i.e. shoots in air), the plants brushed with dilute Triton X
357 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 CO₂ uptake for underwater photosynthesis in light, and O₂ uptake for respiration in darkness; both elevate shoot pO₂ 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 CO₂ entry for underwater P_N (Fig. 5) and in darkness O₂ entry into leaves and internal diffusion along the petiole (Fig. 4). Interestingly, the

measurements of underwater P_N also showed that leaves of plants with roots pre-exposed to salinity had higher rates than leaves from non-saline plants (Fig. 5). This effect of pre-exposure to salinity was visually associated with more prominent gas films on these leaves when submerged. Further studies are needed to determine how any salt-induced structural or chemical alterations of the cuticle might influence leaf hydrophobicity and leaf gas film formation/persistence upon submergence. Differences in leaf hydrophobicity, as dependent on growth conditions, have been described for other species (seasonal changes in hydrophobicity observed in species of e.g., beech, oak and ginkgo; Neinhuis & Barthlott 1998).

Leaf gas films of *M. siculus* protected against ion intrusion and facilitated gas exchange under saline submergence – but did this contribute to survival and growth of the plants? Plants with gas films did not grow during submergence, even in non-saline water, but when the gas films were removed shoot DM was reduced to half of the initial value (Fig. 6) as older leaves were injured, which then presumably was the cause of these leaves being detached within 3 d of submergence (data not shown). Plants in saline submergence solution suffered the most when leaf gas films were removed, degradation of the shoot was substantial (RGR was -0.3 d^{-1}) presumably due to the high tissue Na^+ and Cl^- concentrations (Fig. 3) having toxic effects. Detrimental effects of high tissue Na^+ and Cl^- concentrations on leaf functioning were evident as a loss of photosynthetic capacity in leaves with gas films removed (Fig. 5). These plants did not survive after de-submergence (Supporting Information Table 1S).

Although *M. siculus* could only survive complete submergence with low salinity ($\leq 50 \text{ mol m}^{-3}$ NaCl), this species tolerated waterlogging with high salinity of 500 mol m^{-3} NaCl (Teakle *et al.* 2012). When *M. siculus* maintains shoot contact with the atmosphere, the continuum of tissue gas-filled spaces (in petioles, stem/root phellem, and primary aerenchyma in roots) promotes O_2 movement into the root system. *M. siculus* with shoots in air can even survive in a severely hypoxic root medium with salinity near that of seawater (Teakle *et al.* 2011). The thick layer of highly porous phellem at the shoot base and extending down the roots (porosity in Supporting Information Table 2S) provides a low-resistance pathway for O_2 transport (i.e. aerenchyma) into and along roots of *M. siculus* (Teakle *et al.* 2011; Verboven *et al.* 2012).

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

462

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469 **Tables**

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

471

Treatment Number	Root-zone salinity (mol m ⁻³ NaCl)	Root-zone aeration	Submergence treatment [#]	Submergence salinity (mol m ⁻³)	Gas film present*
<i>Dose response experiment</i>					
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
<i>Leaf gas films – influence on ion intrusion, underwater photosynthesis and internal aeration</i>					
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
<i>Additional growth conditions to establish supporting information</i>					
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

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

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

Submergence treatment (mol m ⁻³ NaCl)	Gas films present	Leaflet Chl _a (μ g g ⁻¹ FM)	Leaflet Chl _b (μ g g ⁻¹ FM)	Leaflet Chl <i>a:b</i>
Emergent 0.1	Yes ¹	456 \pm 68	103 \pm 14	4.4 \pm 0.1
Emergent 0.1	No ²	365 \pm 37	81 \pm 9.1	4.5 \pm 0.1
Emergent 100	Yes ¹	249 \pm 21	56 \pm 1.6	4.4 \pm 0.3
Emergent 100	No ²	241 \pm 23	52 \pm 5.2	4.6 \pm 0.04
Submerged 0.1	Yes	472 \pm 21	119 \pm 4.1	4.0 \pm 0.06
Submerged 0.1	No	471 \pm 20	117 \pm 5.5	4.0 \pm 0.06
Submerged 100	Yes	338 \pm 38	83 \pm 9.2	4.1 \pm 0.03
Submerged 100	No	196 \pm 22	58 \pm 6.1	3.4 \pm 0.15
<i>LSD</i> _{0.05}		75.6	17.6	<i>n.s.</i>

483 ^{1,2}Emergent plants do not possess a ‘gas film’, i.e. ¹brushed with DI water and ²brushed with 0.1%
 484 v/v Triton X, and the shoots remained in air.

485 **Figure legends**

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

490

491 **Figure 2.** Shoot ion concentrations of *Melilotus siculus* in response to increasing levels of salinity
492 combined with submergence for 7 d (dose response experiment). Plants were submerged 28 d after
493 imbibition. Prior to submergence, all plants had been pre-treated in the root medium with either 0.1,
494 25, 50 or 100 mol m⁻³ NaCl for 7 d with the last 4 d in stagnant deoxygenated nutrient solution (the
495 various root-zone conditions continued during each respective submergence treatment). Leaf gas
496 films were present on all plants and were not artificially manipulated in this experiment. Values are
497 the mean (±SE, n=4). Aerated controls for 0.1 and 100 mol m⁻³ NaCl treatments were (μmol g⁻¹
498 DM): Cl⁻, 56 ± 4.6 and 872 ± 65; Na⁺, 165 ± 4.4 and 1363 ± 53; K⁺, 1512 ± 85 and 1072 ± 50
499 (mean ±SE, n=4).

500

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

509

510 **Figure 4.** O₂ dynamics of petioles of *Melilotus siculus* in response to submergence in the dark and
511 the influence of leaf gas films. O₂ microelectrodes were inserted 50 mm below the leaflets, with the
512 petiole in stagnant deoxygenated 0.1% w/v agar submergence medium. Gas films were removed by
513 brushing with 0.1% v/v Triton X (-GF). Plants with gas films intact were brushed with DI water
514 (+GF). Values are the mean ± SE (n=8). Different letters indicate significant differences between
515 treatments (P<0.05) based on Tukey's test.

516

517 **Figure 5.** Underwater net photosynthesis (P_N) of *Melilotus siculus* in response to submergence in
518 non-saline or saline solution and with presence or removal of leaf gas films. Plants were submerged
519 28 d after imbibition. Prior to submergence, all plants had been pre-treated in the root medium with
520 0.1 or 100 mol m⁻³ NaCl for 7 d with the last 4 d in stagnant deoxygenated nutrient solution (the
521 various root-zone conditions continued during each respective submergence treatment). Gas films
522 were removed by brushing with 0.1% v/v Triton X (-GF). Plants with gas films intact were brushed
523 with DI water (+GF). Leaflets were removed 3 d after submergence treatments commenced and
524 underwater P_N measured for samples from plants that had been with shoots in air or submerged
525 (with or without gas films). Values are the mean (\pm SE, $n=4$). Different letters indicate significant
526 differences between treatments ($P<0.05$) based on Tukey's test. Aerial photosynthesis was
527 measured on intact emergent plants (i.e. shoots in air) and the average value for young fully
528 expanded leaves in air was $20.6 \pm 1.7 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$.

529

530 **Figure 6.** Shoot dry mass (DM, a) and shoot relative growth rate (RGR, b) of *Melilotus siculus* in
531 response to submergence with gas films intact (+GF) or removed (-GF) in saline and non-saline
532 solutions for 6 d. Plants were submerged 28 d after imbibition. Prior to submergence, all plants had
533 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
534 stagnant deoxygenated nutrient solution (the various root-zone conditions continued during each
535 respective submergence treatment). Gas films were removed by brushing with 0.1% v/v Triton X (-
536 GF). Plants with gas films intact were brushed with DI water (+GF). Values are the mean (\pm SE,
537 $n=4$). Different letters represent a significant difference between treatments ($P<0.05$) based on
538 Dunn's post hoc test. Values for root DM did not change during the treatment period and were not
539 significantly different between treatments (average 0.127 g). DM for the emergent plants (i.e. with
540 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);
541 100 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 O₂ 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 CO₂ and O₂ 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°C. *Australian Journal of Chemistry* **20**, 2017-2023.
- Marañón 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.

- Munns R. & Tester M. (2008) Mechanisms of salinity tolerance. *Annual Review of Plant Biology* **59**, 651-681.
- Munns R., Wallace P.A., Teakle N.L. & Colmer T.D. (2010) Measuring soluble ion concentrations (Na^+ , K^+ , Cl^-) in salt-treated plants In: *Plant Stress Tolerance: Methods and Protocols* (ed R. Sunkar), pp. 371-382. Springer, Heidelberg.
- Neinhuis C. & Barthlott W. (1997) Characterization and distribution of water-repellent, self-cleaning plant surfaces. *Annals of Botany* **79**, 667-677.
- Neinhuis C. & Barthlott W. (1998) Seasonal changes of leaf surface contamination in beech, oak, and ginkgo in relation to leaf micromorphology and wettability. *New Phytologist* **138**, 91-98.
- Pang J.Y., Newman I., Mendham N., Zhou M. & Shabala S. (2006) Microelectrode ion and O_2 fluxes measurements reveal differential sensitivity of barley root tissues to hypoxia. *Plant, Cell & Environment* **29**, 1107-1121.
- Pedersen O. & Colmer T.D. (2012) Physical gills prevent drowning of many wetland insects, spiders and plants. *Journal of Experimental Biology* **215**, 705-709.
- Pedersen O., Colmer T.D. & Sand-Jensen K. (2013) Underwater photosynthesis of submerged plants – recent advances and methods. *Frontiers in Plant Science* **4**.
- Pedersen O., Rich S.M. & Colmer T.D. (2009) Surviving floods: leaf gas films improve O_2 and CO_2 exchange, root aeration, and growth of completely submerged rice. *The Plant Journal* **58**, 147-156.
- Pedersen O., Rich S.M., Pulido C., Cawthray G.R. & Colmer T.D. (2011) Crassulacean acid metabolism enhances underwater photosynthesis and diminishes photorespiration in the aquatic plant *Isoetes australis*. *New Phytologist* **190**, 332-339.
- Plett C.D. & Møller I.S. (2010) Na^+ transport in glycophytic plants: what we know and would like to know. *Plant, Cell & Environment* **33**, 612-626.
- Raskin I. (1983) A method for measuring leaf density, thickness and internal gas. *Hortscience* **18**, 698-699.
- Raskin I. & Kende H. (1983) How does deep water rice solve its aeration problem? *Plant Physiology* **72**, 447-454.
- Rogers M.E., Colmer T.D., Frost K., Henry D., Cornwall D., Hulm E., Deretic J., Hughes S.R. & Craig A.D. (2008) Diversity in the genus *Melilotus* for tolerance to salinity and waterlogging. *Plant and Soil* **304**, 89-101.
- Setter T.L., Waters I., Wallace I., Bekhasut P. & Greenway H. (1989) Submergence of rice. I. Growth and photosynthetic response to CO_2 enrichment of floodwater. *Australian Journal of Plant Physiology* **16**, 251-263.
- Shabala S.N. & Mackay A. (2011) Ion transport in halophytes. In: *Advances in Botanical Research* (eds J.C. Kader & M. Delseny), pp. 151-199. Academic Press, Burlington.
- Teakle N.L., Armstrong J., Barrett-Lennard E.G. & Colmer T.D. (2011) Aerenchymatous phellem in hypocotyl and roots enables O_2 transport in *Melilotus siculus*. *New Phytologist* **190**, 340-350.
- Teakle N.L., Bowman S., Barrett-Lennard E.G., Real D. & Colmer T.D. (2012) Comparisons of annual pasture legumes in growth, ion regulation and root porosity demonstrate that *Melilotus siculus* has exceptional tolerance to combinations of salinity and waterlogging. *Environmental and Experimental Botany* **77**, 175-184.
- Teakle N.L. & Tyerman S.D. (2010) Mechanisms of Cl^- transport contributing to salt tolerance. *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., Nicolai B.M., Colmer T.D. & Teakle N. (2012)
 639 Root aeration via aerenchymatous phellem: three-dimensional micro-imaging and radial O₂
 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 O₂.
 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.¹ Values are the mean (\pm 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 \pm 0.011 ^{de}	0.157 \pm 0.038 ^d
Emergent 0.1	No ²	0.364 \pm 0.022 ^d	0.149 \pm 0.022 ^d
Emergent 100	Yes ¹	0.244 \pm 0.044 ^d	<i>n.d.</i>
Emergent 100	No ²	0.228 \pm 0.025 ^d	<i>n.d.</i>
Submerged 0.1	Yes	0.031 \pm 0.009 ^c	0.373 \pm 0.085 ^d
Submerged 0.1	No	-0.129 \pm 0.038 ^{ab}	0.282 \pm 0.029 ^d
Submerged 100	Yes	-0.026 \pm 0.020 ^b	*
Submerged 100	No	-0.194 \pm 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. ¹brushed with DI water and ²brushed 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 (\pm 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 \pm 1.1	31.5 \pm 2.2	0.48 \pm 0.07	0.92 \pm 0.2
Submerged	0	4.5 \pm 1.1	16.3 \pm 4.3	0.078 \pm 0.005	0.10 \pm 0.04
Emergent	100	6.7 \pm 0.7	28.9 \pm 1.06	0.45 \pm 0.01	1.06 \pm 0.1
Submerged	100	2.0 \pm 0.4	7.9 \pm 1.1	0.11 \pm 0.01	0.19 \pm 0.1
<i>P-value sub x salt*</i>		0.369	0.038	0.405	0.361
<i>LSD</i> _{0.05}		2.2	3.2	0.05	0.12

B.

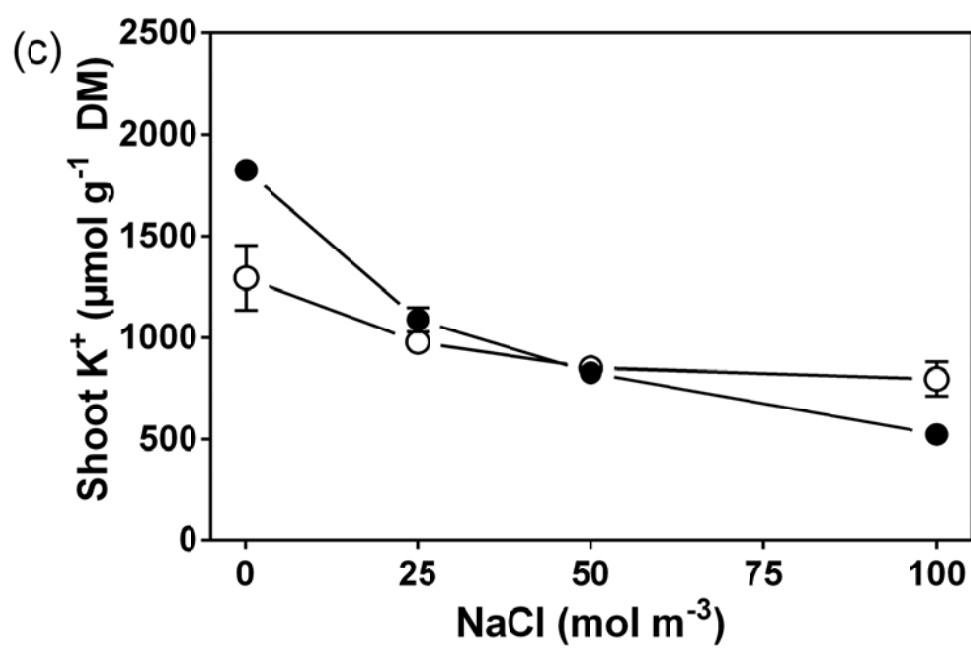
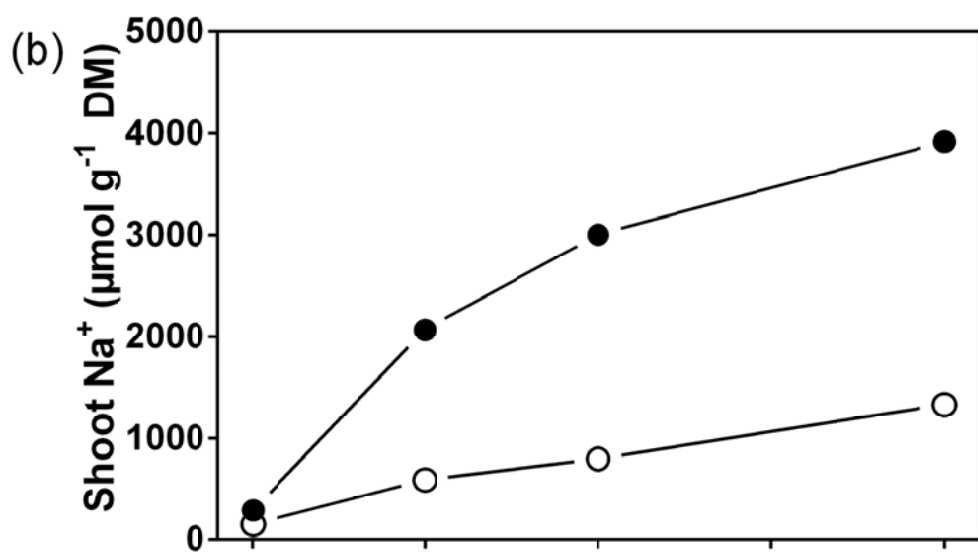
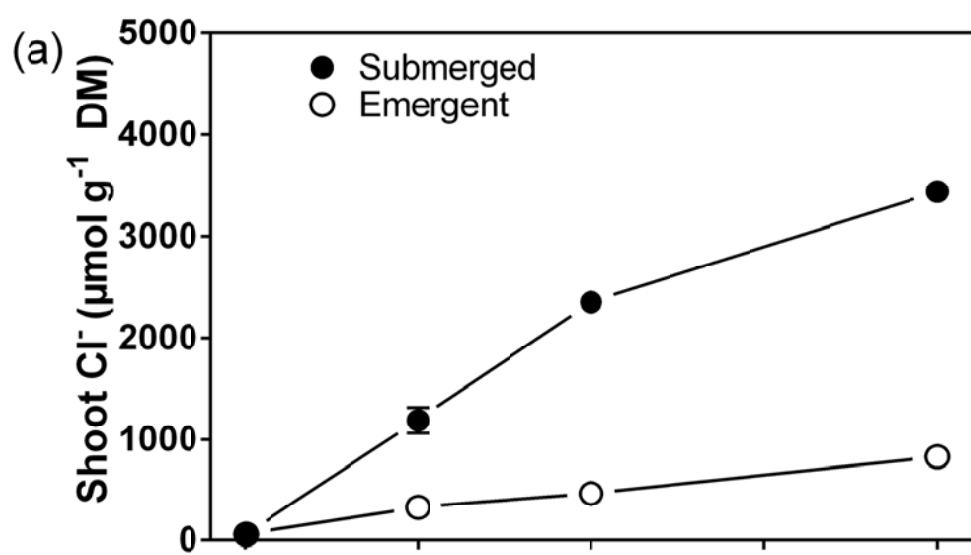
Submergence treatment	Salinity (mol m ⁻³)	Shoot concentration (μ mol g ⁻¹ DM)		
		Cl ⁻	Na ⁺	K ⁺
Emergent	0	57 \pm 4	148 \pm 1	1293 \pm 160
Submerged	0	102 \pm 6	288 \pm 17	1824 \pm 41
Emergent	100	826 \pm 38	1331 \pm 76	795 \pm 84
Submerged	100	3440 \pm 37	3918 \pm 69	523 \pm 14
<i>P-value sub x salt*</i>		<0.001	<0.001	<0.001
<i>LSD</i> _{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



Tissue concentration

