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Plumbing the depths: extracellular water storage in specialized leaf structures and its functional expression in a three-domain pressure-volume relationship

1   **Plumbing the depths: extracellular water storage in specialized leaf structures and its**

2   **functional expression in a three-domain pressure-volume relationship**

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5   **Running title: Three-domain pressure-volume relationship reflects leaf structure**

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9   **[1]Hoa T. Nguyen, [1,3]Patrick Meir, [2]Joe Wolfe, [3,4]Maurizio Mencuccini and [1]Marilyn C. Ball**

10   [1]Plant Science Division, Research School of Biology, The Australian National University, Acton, ACT

11   2601, Australia

12   [2]School of Physics, University of New South Wales, Sydney, NSW 2052, Australia

13   [3]School of GeoSciences, University of Edinburgh, Crew Building, West Mains Road, Edinburgh EH9

14   3JN, UK

15   [4]ICREA at CREAF, Universidad Autonoma de Barcelona, Cerdanyola del Valles, Barcelona, Spain

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17

18   Authors for correspondence:

19   Hoa T. Nguyen

20   Email: hoa-thi.nguyen@anu.edu.au

21

22   Marilyn C. Ball

23   Email: marilyn.ball@anu.edu.au

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27 **Abstract:**

28 A three-domain pressure – volume relationship (PV curve) was studied in relation to leaf anatomical

29 structure during dehydration in the grey mangrove, *Avicennia marina*. In domain 1, relative water

30 content (RWC) declined 13% with 0.85 MPa decrease in leaf water potential, reflecting a decrease in

31 extracellular water stored primarily in trichomes and petiolar cisternae. In domain 2, RWC decreased

32 by another 12% with further reduction in leaf water potential to -5.1 MPa, the turgor loss point. Given

33 the osmotic potential at full turgor (-4.2 MPa) and the effective modulus of elasticity (~40 MPa),

34 domain 2 emphasized the role of cell wall elasticity in conserving cellular hydration during leaf water

35 loss. Domain 3 was dominated by osmotic effects and characterized by plasmolysis in most tissues

36 and cell types without cell wall collapse. Extracellular and cellular water storage could support an

37 evaporation rate of 1 mmol $m^{-2}s^{-1}$ for up to 54 and 50 min, respectively, before turgor loss was

38 reached. This study emphasizes the importance of leaf anatomy for the interpretation of PV curves,

39 and identifies extracellular water storage sites that enable transient water use without substantive

40 turgor loss when other factors, such as high soil salinity, constrain rates of water transport.

41

42 **Key words:** mangrove, PV curve, leaf structure, dehydration, plasmolysis, extracellular water,

43 cisternae, trichomes, leaf water capacitance.

2

Introduction

Increasing vulnerability to drought is a major global concern as rise in average temperature is
associated with increase in the frequency and intensity of drought in many locations (Reichstein *et al.*,
2013). Drought - induced tree mortality has been recorded worldwide in diverse ecosystems and
climatic zones in the past few decades (Allen *et al.*, 2015). These reports range across a continuum of
drought tolerance from species as drought sensitive as those growing in tropical rainforests (Rowland
et al., 2015) to the highly drought and salinity tolerant species of mangrove forests (Lovelock *et al.*,
2009). Drought-induced mortality has been linked with stem hydraulic deterioration (Anderegg *et al.*,
2012, Rowland *et al.*, 2015). Determination of stem vulnerability to drought can be difficult, especially
in trees. However, Zimmermann (1983) suggested that leaves should be more vulnerable to hydraulic
dysfunction than stems, as shown, for example, in sugar maple (Choat *et al.*, 2005). Indeed, leaf
traits, such as the turgor loss point, can provide a powerful means of identifying species that are most
vulnerable to drought (Bartlett *et al.*, 2012).

There is an urgent need to better understand the interplay of leaf water relations and leaf structure.
Most recent studies have focussed on correlations between hydraulic anatomy and hydraulic
conductance that inform our understanding of morphological constraints on carbon gain in relation to
water loss by leaves under different environmental conditions (Sack & Scoffoni, 2013). For example,
interspecific differences in vein and stomatal densities are associated with differences in leaf hydraulic
conductance, and hence also in capacities for photosynthetic gas exchange under conditions of both
high and low water availability (Brodribb & Holbrook, 2003). However, much less is known about the
influence of other aspects of leaf anatomy on leaf water relations as measured by pressure/volume
relationships.

The pressure-volume method is used to analyse the water content of a leaf in terms of water potential
and its components (Scholander *et al.*, 1964, Tyree & Hammel, 1972, Cheung *et al.*, 1975). For a leaf
or tissue in a pressure chamber, with the petiole protruding through a seal into air at atmospheric
pressure, a plot of the pressure in the chamber vs. the volume of sap expressed from the petiole or
the residual weight of the leaf (hereafter called the PV curve) yields a relationship which is usually
reported as having two domains with differing behaviours. In these two-domain examples, when the

74   leaf water potentials are relatively high, decline in water content with increasing pressure in the

75   chamber is dominated by quasi-elastic shrinkage of the cells as the turgor pressure is reduced. This

76   first domain ends when turgor falls to near zero (i.e. intracellular osmotic pressure approximately

77   equals the air pressure in the chamber). Further reduction in cell water content is determined mainly

78   by the osmotic behaviour of the flaccid cell and is often approximated by the van't Hoff law. Thus the

79   PV curve is information-rich, enabling the calculation and functional evaluation of parameters such as

80   leaf capacitance for water storage (**Q**) (Tyree & Ewers, 1991), the osmotic potentials at full turgor

81   (Ψ_π^{100}) and at the turgor loss point (Ψ_π^{0}), and an effective volumetric modulus of elasticity (**ε**), all of

82   which are strongly associated with interspecific variation in drought tolerance (Bartlett *et al.*, 2012).

83

84   The theory underpinning interpretation of PV curves was developed with the approximation that

85   leaves behave like osmometers (Scholander *et al.*, 1965) and that an average behaviour can be

86   described for all cells in a leaf. However, two aspects of leaf structural complexity affect interpretation

87   of PV curves. First, leaves are composed of a diverse range of cells specialised in different aspects of

88   function. These cell types are structurally distinct, displaying, for example, differences in cell size and

89   in the thickness and composition of cell walls, each of which could affect their modulus of elasticity,

90   and hence maintenance of turgor during desiccation. In addition, even within a given cell type,

91   variation in solute composition among adjacent hydrated cells (McCully *et al.*, 2010) could affect the

92   osmotic potentials of individual cells at full turgor, and hence also affect the water potentials at which

93   turgor is lost (Bartlett *et al.*, 2012). Such differences between cell types and between cells of a given

94   type could explain the curvilinear transition between the linear elastic and osmotic domains of the PV

95   curve (Cheung *et al.*, 1975). Indeed, Melkonian *et al.* (1982) suggested that the departure from

96   linearity of the elastic domain of the PV curve signals the onset of turgor loss in the most vulnerable

97   cells within a leaf; similarly, Cheung *et al.* (1975) suggested that the turgor loss point estimated from

98   the onset of osmotic behaviour in the PV curve represents the extreme condition when all cells have

99   lost turgor.

100

101   A second complication to interpretation of PV curves arises from the distribution of water in symplastic

102   and apoplastic spaces within leaves. The symplastic space consists of the intracellular components of

103   living cells bounded by the plasma membranes. The apoplastic space is more difficult to define.

104 Canny (1995) recognized four different appoplastic spaces: the lumina of xylem conduits and fibers,

105 the gaseous intercellular space, and the water and Donnan free spaces of cell walls. However, more

106 spaces may be identified as functional leaf anatomy becomes better understood. Differentiation of

107 symplastic and apoplastic water is important in the estimation of PV parameters, such as elasticity.

108

109 In some species, PV curves with more than two domains have also been reported. The temporary

110 accumulation of water in apoplastic spaces has given rise in some studies to a third domain at the

111 highest levels of hydration in a PV curve. For example, in sphagnum moss, an initial domain largely

112 corresponded to the loss of water stored in the specialised, dead, hollow hyaline cells (Hajek &

113 Beckett, 2008). In this domain, sphagnum moss shoots lost up to 70% of water content without a

114 measurable change in shoot water potential. Once this extracellular water was depleted, then the PV

115 curves of drying shoots showed the two successive domains dominated by turgor reduction and

116 osmotic relationships (Hajek & Beckett, 2008). Similar patterns were observed in other poikilohydric

117 species of moss, lichen, liverworts, and a filmy fern (Beckett, 1997). A similar domain has also been

118 reported in more complex homoiohydric species, such as white oak (Parker & Pallardy, 1987),

119 *Pseudotsuga menziesii* (Mirb.) Franco (Kubiske & Abrams, 1991), and olive (Dichio *et al*., 2003), but

120 interpreted as an artefact of rehydration, also known as "the plateau effect" (Parker & Pallardy, 1987).

121

122 In the present study, the nature of the PV curve was related to leaf anatomy in *Avicennia marina*

123 subsp. *australasica* (Walp.) J. Everett, a highly salt-tolerant mangrove species (Naidoo *et al*., 2011,

124 Nguyen *et al*., 2015). Leaves of *A. marina* naturally endure enormous variability in hydration with leaf

125 water potentials ranging from -0.1 MPa during rainfall events to -6.0 MPa during low tides under hot,

126 dry weather (Constable, 2014, Walker, 2014). The leaf structure of *A. marina* is typical of C_3 species

127 in having layers of palisade and spongy mesophyll. In addition, *A. marina* has salt secretion glands, a

128 trichome layer on the abaxial surface, and a hypodermal water storage layer, similar to many other

129 halophytes (Hutchings & Saenger, 1987). Thus *A. marina* is ideal for our study because it is a widely

130 distributed species with leaves that are both highly tolerant of drought and possess complex cellular

131 structures.

132

133 **Materials and Methods**

5

134 **Plant materials**

135 One branch was collected from each of five co-occurring trees of *A. marina* subsp. *australasica*

136 growing naturally along the Clyde River, Batemans Bay, New South Wales, Australia, and brought

137 back to the lab in a black plastic bag stored in an insulated box to prevent water loss. A hand-held

138 refractometer (A.S.T. Co. Ltd., Japan) was used to measure salinity of surface water collected at high

139 tide and soil water collected at low tide from a soil depth of 30 cm. Soil water was extracted with a

140 suction device (McKee, 1993) from three locations around each tree and measurements of these

141 three salinities were averaged to give one value per tree (n = 5 trees).

142

143 **PV curve**

144 Upon return to the lab, the cut ends of all branches were recut under a solution of 25 mM NaCl and

145 transferred to a bucket, keeping the cut ends under solution and enclosing the bucket to keep the

146 humidity high around the leaves. The branches were allowed to rehydrate overnight in the dark in a

147 cold room at 5°C. The composition of this perfusion solution matched average salt concentrations

148 measured in the xylem sap of stems and leaves from *Avicennia marina* grown in seawater salinity

149 under laboratory (Ball, 1988) and natural field conditions (Stuart *et al.*, 2007). Before PV curves were

150 constructed, leaves were cut from branches near the petiole – stem junctions while the petioles were

151 submerged in perfusion solution. Leaves were then transferred to a beaker, keeping the petioles

152 under solution. The beaker was covered with plastic wrap to maintain a high humidity around the

153 leaves and they were allowed to fully hydrate in the dark at room temperature for at least 30 min. The

154 leaves were then dabbed dry with tissue paper to remove excess water before measuring leaf fresh

155 mass (XP 205 Metter Toledo balance, Mettler - Toledo Ltd., Greifensee, Switzerland) and leaf water

156 potential (Pressure chamber model 1050D, PMS Instrument, Albany, USA).

157

158 Five PV curves were constructed, each made with one leaf from one tree. Leaf water potential was

159 measured at intervals corresponding to decreases in leaf fresh mass of 5-10 mg as the leaves air-

160 dried at room temperature. Time intervals between measurements ranged from 1 min at the start of

161 dehydration to 40 min for the final measurements, made when leaf water potentials reached values

162 between -5 and -6 MPa, corresponding to the minimal values Constable (2014) measured during

163 diurnal periods under field conditions in the same population of trees as in the present study. The leaf

6

164   perimeter with petiole excluded was traced on paper for leaf area measurement with a LI 3100 area

165   meter (LICOR Inc., Lincoln, Nebraska, USA). Leaf dry mass was recorded after 48 h oven-drying to

166   constant mass at 70°C. PV curve parameters were calculated as in Table 2 and other particulars are

167   given in the results.

168

169   **Leaf anatomy**

170   **Light microscopy**

171   Two opposite, mature leaves were selected for anatomical analyses from the same branches that

172   provided leaves for measurement of PV curves. One leaf in each pair was used for measurements of

173   the tissue composition and trichome volume. Trichomes were approximated as cylinders, and

174   trichome volumes were estimated based on height and internal lumen diameter. Transverse sections

175   (20 - 30 μm thickness) were cut from the petiole and leaf blade with a sledge microtome (GSL 1, S.

176   Lucchinetti, Schenkung Dapples, Zürich, Switzerland) and stained with either Toluidine blue (0.05%

177   w/v in water) or a 50:50 mixture of Alcian Blue (1% w/v in 50% v/v alcohol) and Safranin O (1% w/v in

178   water). The matching leaf in each pair was used to estimate trichome density, which required an

179   inside-out epidermal preparation. The leaf epidermis was loosened by incubating the leaf in

180   maceration solution (five parts 30% v/v hydrogen peroxide to one part glacial acetic acid) at 70°C for

181   at least 2 h. Then the abaxial epidermis was removed with forceps, washed in water, and stained as

182   above. Micrographs were taken at a range of magnifications using an upright microscope (DM 6000,

183   Leica, Wetzlar, Germany) equipped with a digital camera (SPOT Flex 64MP Color FireWire 15.2,

184   Diagnostic instruments.inc, USA) and analysed using ImageJ (Rasband, 1997 - 2014).

185

186   **Cryo- Scanning Electron Microscopy (cryo-SEM)**

187   PV curves were measured, and then leaf anatomical features, including the distribution of gas, liquid

188   water, and cell structure, were visualized at different states of hydration using cryo – Scanning

189   Electron Microscopy (cryo-SEM) according to McCully *et al.* (2009). One branch with four twigs

190   similar in size was selected from each of three of the five study trees, then cut under perfusion

191   solution (25 mM NaCl) and left for a few hours until fully hydrated. Then the four twigs, each with at

192   least three pairs of fully expanded leaves, were cut under perfusion solution from each branch. One

193   twig from each branch was sampled immediately upon removal from perfusion solution and the

194 remaining three twigs of each branch were then air-dried for differing periods of time before sampling

195 to capture change in leaf structure associated with key features of the previously measured PV

196 curves. At each sampling time, one leaf in each pair was used to measure leaf water potential while

197 the other leaf was frozen rapidly (less than 0.1 s) with cryo-pliers cooled with liquid N_2 (LN2) (McCully

198 *et al.*, 2010). Frozen pieces (1 - 2 x 0.5 cm) were cut from the desired region under LN2, placed in

199 cryo-vials and stored in LN2 until processed for cryo-SEM. Then, frozen leaf segments were trimmed

200 to smaller pieces (3 mm length) under LN2, planed with a cryo-microtome (Leica EM FC7, Leica,

201 Wetzlar, Germany) and visualised by cryo-SEM (Hitachi 4300SE/N, Hitachi High Technologies,

202 Japan) following McCully *et al.* (2010).

203

204 **Mucilage Analysis**

205 Sample preparation

206 Gel extruded from petioles of *A. marina* was collected and dispersed in 5% EtOH/water. The solution

207 was centrifuged to remove insoluble extraneous matter and the supernatant freeze-dried.

208

209 Polysaccharide analysis (% composition)

210 The reductions of the constituent uronic acid methyl esters and the free uronic acids were carried out

211 following the protocol of Kim and Carpita (1992) and of Pettolino *et al.* (2012). The reduced

212 polysaccharides were then hydrolysed, reduced, acetylated and subject to gas chromatography mass

213 spectrometry (GC/MS) analysis as described by Peng *et al.* (2000).

214

215 **Water uptake by trichomes**

216 Three twigs, each with a fully mature leaf pair, were harvested from three saplings of *A. marina* that

217 had been hydroponically grown (nutrient solution salinized with addition of 250 mM NaCl) from

218 propagules that originated from the same field populations used for PV curve analyses. Leaves were

219 rehydrated and then air-dried, with water potential determined at intervals during drying as described

220 above for PV curves. Following each measurement of leaf water potential, leaves were tested for

221 uptake of water by the trichome layer that covered the abaxial leaf surface. Upon removal from the

222 pressure chamber, a leaf was positioned to view the trichome layer under an epi-fluorescence

223 microscope (Zeiss Axiostar plus, Carl Zeiss, Germany). A drop of 0.1% fluorescein sodium salt

224 solution (British Drug Houses, Poole, England) was applied to the trichome surface and fluorescence

225 from the dye was observed by standard methods under blue-exciting light. A threshold for water

226 uptake by trichomes was determined by the highest leaf water potential at which dye applied to the

227 abaxial leaf surface disappeared into the trichomes. This threshold was used to estimate the onset of

228 drainage from trichomes as well-hydrated leaves were air-dried.

229

230 **Data analysis**

231 Data analyses using simple linear regression or one-way ANOVA were performed in Genstat version

232 16 (Payne, 2014). Data did not require transformation prior to analysis. Where relationships were

233 significant, differences between treatment means were tested *post hoc* for significance (here

234 considered $P \leq 0.05$) using Fisher's Least Significant Difference test. Unless otherwise stated, all

235 results given in the text are mean values $\pm$ standard error (se), n = 5.

236

237 **Results**

238

239 **PV curve analysis**

240 All measurements were made on leaves collected from mature trees that grew naturally where soil

241 water salinity at a depth of 30 cm averaged 49 ± 0.5 ppt (parts per thousand by weight) and surface

242 water at high tide averaged 35 ppt. For reference, standard seawater has a salinity of 35 ppt, and an

243 osmotic potential of -2.4 MPa. Thus the roots were exposed to soil and surface water with an osmotic

244 potential ranging from -3.4 to -2.4 MPa, respectively. However, surface water salinity can become

245 higher during low tide when drying concentrates salt at the soil surface.

246

247 Characteristics of the leaves used for PV curve measurements are summarised in Table 3. Fully

248 expanded, sun leaves of *A. marina* had an average area of 14.8 ± 1.1 cm^2 and contained 0.66 ± 0.05

249 g of water when fully hydrated. Relative water content (RWC) was measured on these leaves as a

250 function of leaf water potential (Ψ_{leaf}) during air-drying. Variation in RWC with Ψ_{leaf} followed a

251 complicated relationship with three domains as shown in Fig. 1a.

252

253 Water relations parameters of *A. marina* leaves were calculated from plots relating water content to

254 water potential (commonly called PV curves) and are summarised in Table 4. Domain 1 of the curve

255 was characterised by rapid decline in RWC produced by relatively small application of pressure in the

256 chamber. On average, RWC declined $13 \pm 1\%$ in this domain, for which there was less than 1 MPa

257 decrease in the inferred leaf water potential. Water potential at the transition from the first to the

258 second domain (Ψ_x) averaged -0.85 ± 0.06 MPa (point **B**, Fig. 1a). Domain 2 covered the largest

259 variation in Ψ_{leaf}: a decrease from -0.85 to -5.1 MPa, five times larger than that of domain 1. Over the

260 second domain, RWC decreased by an average of $12 \pm 3\%$, almost as much as that of domain 1.

261 Finally, domain 3 was characterized by more rapid rate of leaf water loss for a given decrease in leaf

262 water potential than the previous domain, domain 2. The transition between domains 2 and 3 (point **C**,

263 Fig.1a) averaged -5.1 ± 0.1 MPa. Given the salinities of pore and surface water, Ψ_{leaf} at this transition

264 was inferred to be the leaf water potential at turgor loss point (Ψ_π^0), which was supported by

265 calculations from the fitted straight line (dashes and points) in Fig. 1b as shown below.

266

267 The data set used in Fig. 1a was replotted in Fig. 1b with the reciprocal of Ψ_{leaf}, ($|1/\Psi_{leaf}|$) plotted as a

268 function of relative water content deficit, calculated with total leaf water content (RWD+, %). The

269 starting point, **c**, of the linear relationship between $|1/\Psi_{leaf}|$ and RWD+ indicated the reciprocal of leaf

270 water potential at the turgor loss point ($|1/\Psi_\pi^0|$); further, the intercept **d_x** is given by the coordinates

271 RWD+ = RWD_x+, i.e. the percentage of water content decrease in domain 1, and $|1/\Psi_{leaf}| = |1/\Psi_\pi^{100}|$,

272 i.e. the reciprocal of the osmotic potential at full turgor. According to these calculations, leaf water

273 potential at the turgor loss point and osmotic potential at full turgor averaged -5.1 ± 0.1 MPa and -4.2

274 ± 0.1 MPa, respectively, in *A. marina* leaves. These calculations excluded extracellular water implied

275 in domain 1. If that water was included, then Ψ_π^{100} would be calculated from the $1/\Psi_{leaf}$ intercept, **d_0**,

276 as shown in Fig. 1b and would average -3.5 ± 0.1 MPa.

277

278 The PV curves were replotted without domain 1, as suggested by Beckett (1997) and Dichio *et al.*

279 (2003) to account for effects of extracellular water (the plateau effect) on calculation of leaf water

280 relations parameters from the PV curve (Supplement S1). Calculation of Ψ_π^0 was unaffected by

281 removal of domain 1, but Ψ_π^{100} was changed because the method of Beckett (1997) and Dichio et al.

282 (2003) redefined the status of a fully hydrated leaf. Specifically, calculation of maximum leaf water

283 content (WC_{max}) changed from (FM_{max} - DM) to (FM_{max} - WC_{ex} - DM), where FM_{max} is the maximum

284 leaf fresh mass, DM is the leaf dry mass, and WC_{ex} is the extracellular water content. As WC_{max}

285 decreased, RWC increased accordingly (see Table 2 for RWC calculation). Upon the removal of

286 domain 1, Ψ_π^{100} was calculated at the $1/\Psi_{leaf}$ intercept of the linear regression between inverse leaf

287 water potential ($|1/\Psi_{leaf}|$, MPa^{-1}) and relative water deficit, calculated without extracellular water,

288 (RWD-, %), i.e. point **d** (Supplement S1b), and averaged -4.2 ± 0.1 MPa. This value was equal to that

289 calculated at $\mathbf{d_x}$ (-4.2 ± 0.1 MPa, P = 0.968) but significantly different from that calculated at $\mathbf{d_0}$ (-3.5 ±

290 0.1 MPa, P <0.001).

291

292 **Calculation of water storage capacitance and bulk modulus of elasticity**

293 The area-specific water storage capacitance (called water storage capacitance hereafter) was

294 calculated as the mass of water lost per unit area per unit decrease in water potential (Table 2). Two

295 values of water storage capacitance were calculated from different points on the PV curve (Table 4).

296 Water storage capacitance of domain 1 ($\mathbf{Q_x}$) was calculated from points **A** to **B** (Fig. 1a) and the

297 capacitance of domain 2 ($\mathbf{Q_e}$) was calculated from points **B** to **C** (Fig. 1a). $\mathbf{Q_x}$ averaged 69 ± 6 g m^{-2}

298 MPa^{-1}, and $\mathbf{Q_e}$ averaged 13 ± 4 g m^{-2} MPa^{-1}. In other words, application of a chamber pressure of 1.0

299 MPa during dehydration in domain 1 would release approximately 69 g water per m^2 leaf area

300 whereas similar application of 1.0 MPa in domain 2 would release about 13 g water per m^2 leaf area.

301 The average storage capacitance over both domains ($\mathbf{Q_g}$, points **A** to **C**, Fig. 1a) was 22 ± 4.8 g m^{-2}

MPa^{-1}.

302

303

304 The effective bulk modulus of elasticity, $\boldsymbol{\varepsilon}$, is defined as the change in chamber pressure or water

305 potential required for a fractional decrease in a cell's water volume ($\boldsymbol{\varepsilon} = \Delta P/(\Delta V/V)$). The

306 compressibility of water over this range of pressures is negligible, so $\boldsymbol{\varepsilon} = \Delta P/\Delta RWC$. The values of $\boldsymbol{\varepsilon}$

307 thus estimated (Table 4) averaged 7 ± 1 and 37 ± 10 MPa for domains 1 ($\boldsymbol{\varepsilon_x}$) and 2 ($\boldsymbol{\varepsilon_e}$), respectively,

308 with an average value, $\boldsymbol{\varepsilon_g}$, of 21 ± 2 MPa calculated over both domains.

309

11

310   Water storage capacitance and elasticity calculated upon the removal of domain 1 averaged 13 ± 2 g

311    $m^{-2} MPa^{-1}$ and 38 ± 4 MPa, respectively, and were not significantly different from those calculated

312   from domain 2, i.e. Q_e and ε_e, (P = 0.383, and P = 0.841, respectively).

313

314   **Leaf structure**

315   **Petiole**

316   The basic features of petioles and leaves were characterised by bright field microscopy (Fig. 2).

317   Petioles (Fig. 2a) of *A. marina* leaves were covered with two types of multicellular trichomes.

318   Trichomes of the first type were clustered together with mucus secretion glands in a groove at the

319   petiole - stem junction. Trichomes of the second type covered the rest of the petiole and leaf abaxial

320   surfaces where the trichomes coexisted with salt secretion glands. These trichomes had a rivet-like

321   shape with a cap cell on top, one to two stalk cells in the middle, and a basal cell at the bottom. The

322   cap and stalk cells were dead with a hollow lumen. Trichomes of both types were transparent;

323   however, they appeared black if cyanobacteria were present.

324

325   The cortex was the largest tissue within the petiole and contained two distinct layers. A layer of

326   collenchyma (6 -7 cells thick) occurred beneath the epidermis, and was underlain by a layer of

327   parenchyma (15 - 16 cells thick). Substantial gas spaces occurred between parenchymal cells. The

328   gas spaces in the petiole cortex extended into the bundle sheath extension of the midvein but not to

329   the higher vein orders. The remaining space in the petiole was occupied by the two fimbrial veins and

330   the central vascular cylinder which contained a circular array of vascular bundles as commonly found

331   in eudicots. However, xylem vessels were not evenly distributed among the vascular bundles, with

332   more vessels occurring in bundles near the abaxial than adaxial sides of the petiole. Finally, the pith

333   occupied the most central space, surrounded by vascular tissue. The pith consisted mainly of tightly

334   packed parenchymal cells interspersed with small phloem bundles.

335

336   **Leaf lamina**

337   Leaves had a reticulate venation system with a prominent midvein as shown in Fig. 2b. A thick cuticle

338   covered the adaxial leaf surface except where salt secretion glands occurred at the base of scattered

339   depressions in the leaf surface. These salt secretion glands were in contact with underlying epidermal

340 and hypodermal cells. The midvein vascular structure was similar to that of the petiole. Nevertheless,

341 a ring of fibers surrounding the central vascular cylinder was more developed in the midvein than in

342 the petiole. In place of the cortex, the midvein was bounded by a bundle sheath extension (BSE) that

343 connected the vascular tissue with the upper and lower epidermis. Leaves of *A. marina* were

344 heterobaric but bundle sheath extensions only occurred at vein orders ranging from 1st to 4th. The

345 midvein was also linked with adjacent mesophyll and hypodermal tissues (Fig. 2c).

346

347 Four major tissues contributed most of the leaf lamina thickness (Table 3). The structure of *A. marina*

348 leaves was typical of C_3 species, with veins embedded between the palisade and spongy mesophyll

349 (Fig. 2c). Spongy mesophyll thickness was about two thirds that of palisade mesophyll. Together, they

350 accounted for 43 ± 1% of total leaf thickness. *Avicennia marina* leaves had an additional hypodermal

351 layer located below the upper epidermis, accounting for about 36 ± 1% of leaf thickness. Trichomes

352 on the abaxial leaf surface were similar in structure to the rivet-like trichomes on the petiole but

353 longer, and accounted for 19 ± 1% of total leaf thickness. Salt secretion glands occurred on both leaf

354 surfaces, but stomata were only on the abaxial surface.

355

356 **Change in leaf structures during drying**

357 Leaves were cryo-preserved during drying to fix the cell structure and the spatial distribution of gas

358 and liquid. Changes in these three components of leaf structure were assessed in relation to the three

359 domains of the PV curve using cryo-SEM. Domain 1 was characterized by the occurrence of

360 extracellular water in the gas spaces of the rivet-shaped trichomes, petiole, bundle sheath extension,

361 and leaf lamina. Extracellular water was not observed in samples taken in domain 2. Changes in cell

362 RWC could not be determined from the images because of variation in cell size and shape between

363 leaves. However, during domain 3, plasmolysis was apparent in most cells, but no cell wall collapse

364 was observed except in the trichomes.

365

366 Trichome water status changed with leaf water status. For reference, general trichome structure is

367 shown in Fig. 3a. Trichome lumina were full of liquid water when leaves were fully hydrated (Fig. 3b).

368 During domain 1, this extracellular water was replaced with gas (Fig. 3d) while the stalk cells of

369 trichomes retained their shape; however, the cap cells were collapsed (Fig. 3c, d). In domains 2 and

370 3, both cap and upper stalk cells were shrivelled and collapsed (Fig. 3e).

371

372 Additional measurements using fluorescence microscopy on fresh materials demonstrated the onset

373 of trichome drainage with dehydration (Fig. 4). When viewed under white light the cap cells of the

374 trichomes formed a continuous surface (Fig. 4a). The hollow stalk cells appeared like dark circular

375 areas centred beneath the cap cells. No structure was visible under blue light showing the absence of

376 detectable auto-fluorescence (Fig. 4b). In contrast, the presence of fluorescein was detected on the

377 leaf surface by bright green fluorescence under blue light (Fig. 4c - f). When trichomes were filled with

378 water (Ψ_{leaf} > 0.1 MPa), fluorescein quickly spread over the leaf surface without loss in fluorescence

379 (Fig. 4c, d and movie 1(Supplement S2)). Drainage of water-filled trichomes was detected when Ψ_{leaf}

380 declined to -0.25 ± 0.03 MPa (n = 3). Under these conditions, fluorescein drops remained where

381 applied for approximately a minute before rapidly disappearing into the trichomes (Fig. 4e, f, and

382 movie 2 (Supplement S3)). The estimated density and volume of trichomes (Table 3) indicated that

383 they could potentially hold up to 10% of total leaf water.

384

385 Petiolar gas spaces also functioned in temporary water storage of well-hydrated leaves, and,

386 therefore, were named cisternae. Due to the complex three-dimensional structure, it was not possible

387 to obtain a reliable estimate of water storage in cisternae from the micrographs. When viewed by

388 bright-field microscopy, longitudinal sections through the petiole revealed greater surface structure

389 delimiting these cisternae than expected (Fig. 5a, b). Closer inspection under cryo-SEM revealed that

390 the walls of these cisternae had a rough surface that appeared coated with a mucus-like substance

391 that formed globular structure, hereafter called droplets, with drying. Analysis of a gel extruded

392 naturally from petioles revealed a composition consistent with mucilaginous polysaccharides (Table

393 5). When leaves were well hydrated (Ψ_{leaf} = -0.1 MPa), most cisternae were full of water but some

394 contained large droplets with diameters averaging 6.8 ± 0.8 µm (n = 20) (Fig. 5c, d). Where the

395 planing knife cut through the ice of a water-filled cistern, the eutectic domains of the ice indicated

396 water of high solute content. At the end of domain 1, no cisternae were filled with water and droplet

397 diameter averaged 4.9 ± 0.3 µm (n = 20) (Fig. 5e), smaller than that observed at higher leaf water

14

398   potentials. As leaf water content decreased over domain 2, very few droplets were found and their

399   average diameter was 2.2 ± 0.3 μm (n = 20) (Fig. 5f).

400

401   In the leaf lamina and midvein, small gas spaces between pith parenchyma and between mesophyll

402   cells were also filled with liquid water but only when leaf water potential was less negative than -0.1

403   MPa (Fig. 6). Where the planing knife cut through the ice of a water-filled intercellular gas space, the

404   general absence of eutectic domains in the ice indicated water of very low solute content, in contrast

405   to that of the cisternae. The boundary of the lamina spaces was not specially coated like that of the

406   cisternae. No gas space or external water was found between hypodermal cells. Due to variability in

407   cell sizes within and between leaves, it was not possible to quantify changes in size of the

408   hypodermal cells over domains 1 and 2.

409

410   In domain 3, a wide variety of cell types, including collenchyma and parenchyma cells in the petiole

411   and the hypodermal, mesophyll cells, and epidermal cells in the leaf lamina, showed similar

412   responses to decreases in leaf water potential below the turgor loss point. Gaps appeared between

413   cell walls and the enclosed cells, as is characteristic of plasmolysis (Fig. 7).

414

415

416   **Discussion**

417   The results of the present study revealed a new twist in an old curve and novel insights into the

418   interpretation of the PV curve in *Avicennia marina*. This PV curve exhibited three domains over the

419   range of hydration in which the leaves naturally function under field conditions, even in a single day

420   (Constable, 2014, Walker, 2014). The three domains of leaf dehydration were characterized by (1) the

421   presence and consumption of extracellular water, (2) decline in turgor, and (3) the occurrence of

422   plasmolysis. Each domain, as illustrated in the summary diagram (Fig. 8), and its implications for leaf

423   function, are discussed in detail below.

424

425   **Domain 1: dominated by variation in extracellular water**

426   The domain of highest hydration was characterised by the presence of extracellular water and its

427   disappearance as RWC decreased by 13% while leaf water potential only declined from -0.1 to -0.85

428 MPa (Fig. 1a, Fig. 8). At the highest hydration level, water filled extracellular spaces, including those

429 between mesophyll cells (Fig. 6c), the hollow cores of trichomes (Fig. 3b), and the petiolar cisternae

430 (Fig. 5c). As the leaf dried, water was replaced by gas in these distinct spaces (Figs. 3d, 5e, f, 6d)

431 over different ranges of water potentials, implying that filling of the spaces occurred under different

432 conditions and depended on different processes.

433

434 Liquid water was only observed in some mesophyll extracellular gas spaces when water potential was

435 less negative than -0.1 MPa. Water storage between mesophyll cells would have a negative impact

436 on gas exchange because diffusion of CO_2 is about 10,000 times slower in water than in air, but the

437 extent of this storage was small and short-lived. The lack of eutectic domains in this extracellular

438 water that had been cryo-preserved indicated that the water had very low solute concentrations. How

439 can we account for movement of liquid water from cells to extracellular gas spaces in the absence of

440 an osmotic gradient? The answer may be similar to processes driving refilling of embolized

441 protoxylem vessels in well-hydrated tissues (Rolland *et al.*, 2015). Once the cells had achieved high

442 water potentials, capillary forces due to the combination of hydrophilic walls and close contact

443 between cells may have driven extracellular water accumulation in confined spaces. This limited filling

444 of extracellular gas spaces in excised leaves was not an artefact as similar observations have been

445 made on *A. marina* leaves under natural conditions when they were collected predawn following

446 exposure to nocturnal wetting events (Constable, 2014). Such accumulation of extracellular water in

447 these well-hydrated leaves presumably occurred through foliar absorption of atmospheric water, even

448 though roots grew in seawater; in other words, the water potential gradient during leaf wetting events

449 was reversed from the atmosphere to the leaves to the soil.

450

451 The presence of low solute concentrations in the extracellular water has important implications for salt

452 tolerance of halophytes. During symplastic water uptake at the roots (Moon *et al.*, 1986), *A. marina*

453 excludes approximately 90 - 95% of salt in soil water from entry to the transpiration stream (Ball,

454 1988). When grown in seawater, *A. marina* typically has 25 mM NaCl in the transpiration stream (Ball,

455 1988, Stuart *et al.*, 2007). In *A. marina*, water and associated salt are distributed across a leaf lamina

456 through the vascular system to vein endings and subsequently dispersed to client cells by symplastic

457 pathways (Fitzgerald & Allaway, 1991). This symplastic control prevents catastrophic salt

458   accumulation in apoplastic spaces and facilitates salt management within a leaf. In *A. marina*, part of

459   the salt transported in the transpiration stream is accommodated in growing cells for osmotic

460   adjustment of the vacuoles while the remainder is exported from the leaves via either the phloem or

461   epidermal salt secretion glands, thereby maintaining a favourable salt balance (Ball, 1988). Thus the

462   observation of low solute concentrations in the extracellular water (Fig 5d) is consistent with strong

463   symplastic control of both water and associated salt distribution within the leaves (Fitzgerald &

464   Allaway, 1991).

465

466   Water-filled trichomes began to empty when Ψ_{leaf} declined below -0.25 MPa (Fig. 4e, f and movie 2

467   (Supplement S3)). In contrast to intercellular spaces in the mesophyll, the trichome lumina appeared

468   to contain a mucilaginous substance. This was suggested by the similarity of the ice texture in water-

469   filled trichomes that had been cryo-preserved (Fig 1b) to that observed in cryo-preserved

470   mucilaginous secretions of root tips (McCully *et al.*, 2009). Such a hydrophilic substance in

471   combination with the highly hydrophilic walls and the small diameter of the hairs could have facilitated

472   water uptake at relatively high water potentials, as might occur with exposure to rain or dew. Indeed,

473   under natural field conditions, Constable (2014) observed trichomes to be full of water when leaves of

474   *A. marina* were wet in the morning. Trichomes seemed to serve as one of the main water storage

475   sites, which could hold up to 10% of total leaf RWC. This storage water could be released to the leaf

476   as leaf water potentials declined with dehydration if water transport into the leaf followed a symplastic

477   pathway through the living basal cells of the trichomes to the underlying cells (Fig. 3a). Similarly,

478   water absorbed by trichomes contributed to the water status of underlying leaf cells in oak leaves

479   (Fernández *et al.*, 2014). Thus, identification of trichomes as a major water storage site in *A. marina*

480   also implies that the trichomes may provide a means for rehydration via foliar water uptake.

481

482   Finally, the present study identified a novel water storage structure, here named the cisternae, which

483   occurred mainly in the petiole and extended into the bundle sheath extension of the midvein near its

484   junction with the petiole. Extracellular water occurred in cisternae until leaf water potentials declined

485   to approximately -0.8 MPa. Unlike the extracellular spaces in the mesophyll and the trichomes, the

486   cisternae were relatively large, completely bounded by the walls of cells comprising the surrounding

487   tissues (Fig. 5a,b), and appeared to contain a combination of mucilage, mostly arabinose, and a

17

488   relatively high concentration of solutes (Table 5, Fig. 5d, f). The latter must have been sufficient to

489   attract water from the surrounding cells into the cisternae when leaf water potentials rose above -0.8

490   MPa. Extension of the cisternae into BSE of the midvein would enhance the reach of their influence

491   on leaf water relations as the BSEs are known to play a role in extravascular water dispersal (Canny,

492   1986, Buckley *et al.*, 2011, Sommerville *et al.*, 2012, Zsogon *et al.*, 2015). Conversely, because the

493   osmotic potential of the cisternae would vary with water content, differential drainage of the cisternae

494   during leaf drying could contribute to the curvature linking domains 1 and 2 in the PV curve.

495

496   **Domain 2: Dominated by variable turgor**

497   The second domain was characterized by 12% loss in leaf RWC as leaf water potentials declined

498   from -0.85 to -5.1 MPa. Decrease in cell volume, except for that in trichomes, was not obvious in our

499   micrographs, possibly due to the small scale of total loss in RWC (i.e. 12% across domain 2, Fig. 1a),

500   combined with high variability in cell sizes (Fig. 8). For example, a 12% change in leaf water content

501   would be equivalent to a 4% change in each dimension of an isotropic cell.

502

503   Moreover, there were no obvious changes in gas spaces between mesophyll cells with decreasing

504   Ψ_{leaf}, consistent with observations of desiccating sunflower leaves (Fellows & Boyer, 1978). Tissue

505   connections in the leaf lamina were maintained as cell walls and membranes also maintained contact

506   during shrinkage (i.e. cytorrhysis). Given the large osmotic potential at full turgor (Ψ_π^{100} = -4.2 MPa)

507   and the high modulus of elasticity, most of the decline in leaf water potential with decrease in RWC in

508   the second domain was associated with decreasing turgor pressure.

509

510   The curvature between domains 2 and 3 may reflect differential turgor loss in different cell types with

511   the turgor loss point representing the extreme case where all cells have lost turgor (Cheung *et al.*,

512   1975). Some cells might lose water more rapidly than others due to their properties or their positions

513   within the leaf. In addition, ε is likely to be itself a function of turgor. For example, the high and

514   constant ε region could represent cell wall stretching, and the lower and variable modulus region

515   could represent change in shape. Thus, the curvature shown linking domains 2 and 3 (Fig. 1) likely

516   reflects the large contribution of different cell types to water relations in *A. marina.* Similar curvature

517   was found in many studies where PV curves were constructed from repeated measurements on a

18

518   single leaf or shoot (Cheung *et al.*, 1975, Meinzer *et al.*, 1986, Parker & Pallardy, 1987). In contrast, a

519   sharp transition between domains 1 and 2 has been observed when the PV curve is constructed from

520   a composite of measurements made on many leaves of a species, such as those from woody tropical

521   rainforest (Brodribb & Holbrook, 2003) or used in theoretical analyses, e.g. Suarez and Sobrado

522   (2000). The convexity of the transition may make a useful tool for comparing hydraulic complexity in

523   leaves grown under different conditions or between different species.

524

525   **Domain 3: Dominated by osmotic behaviour of flaccid cells**

526   Domain 3 was characterised by plasmolysis (Figs. 7, 8) as leaf water potentials declined below the

527   turgor loss point once RWC decreased below approximately 75% (or below about 86% if the

528   extracellular water was not included in the accounting) (Table 4). In most living cell types, connections

529   between tissues or between cells comprising a tissue were maintained during desiccation-induced

530   shrinkage. No collapse of cell walls was observed. However, separation between cell walls and

531   plasma membranes developed when Ψ_{leaf} was about 1 MPa lower than Ψ_π^0. Plasmolysis in *A. marina*

532   was also noticed in the field when Ψ_{leaf} was as low as -6 MPa, and the cells recovered following

533   nocturnal rehydration (Constable, 2014). The fact that *A. marina* leaves could recover after reaching

534   the turgor loss point was consistent with a study by Brodribb and Holbrook (2003) which showed that

535   irreversible damage to PSII system only occurred in woody tropical rainforest species at Ψ_{leaf} much

536   lower than Ψ_π^0.

537

538   **Ecophysiological implications of the new PV curve to *A. marina* function**

539   The results of the current study emphasized the importance of leaf anatomy to leaf water relations

540   with far reaching implications for leaf function under field conditions. Previous studies have referred to

541   the first domain as "the plateau effect" (Parker & Pallardy, 1987, Dichio *et al.*, 2003), attributed to

542   artefactual rehydration. The presence of domain 1 due to extracellular water does not change values

543   of PV curve parameters, including Ψ_π^0, Ψ_π^{100}, **ɛ**, and **Q**, as long as those parameters are calculated

544   using data from appropriate domains as shown in Fig. 1 and indicated in Table 4, consistent with

545   recommendations to account for "the plateau effect" (Parker & Pallardy, 1987). However, dismissal of

546   domain 1 as an artefact of rehydration would lead to false assumptions about leaf functions in *A.*

547   *marina* that are inconsistent with field observations and leaf anatomy.

548

549   The PV curves revealed a high modulus of elasticity in leaves of *A. marina*, which plays a major role

550   in cell function over the whole range of water potentials naturally experienced by these leaves. At

551   maximum hydration, leaf water potentials were very high (i.e. -0.1 MPa) while osmotic potential at full

552   turgor was -4.2 MPa (Fig. 1b). The turgor pressure would therefore have been around 4.1 MPa, but

553   there was no evidence that cells burst under these conditions. Presumably, the high modulus of

554   elasticity, i.e. 37 MPa (Table 4), contributed to cell survival when the leaves were fully hydrated.

555

556   Previous studies have interpreted the role of a high ε on leaf function under low water potentials in

557   two ways. First, high ε was proposed to enhance water uptake into a transpiring leaf by increasing

558   the water potential gradient between the leaf and the soil (Bowman & Roberts, 1985). However,

559   Bartlett *et al.* (2012) argued that this proposal disregards the role of hydraulic conductance in

560   determining the water potential gradient. Indeed, studies of diurnal gas exchange in the same

561   population of *A. marina* as the present study showed that reduction in leaf hydraulic conductance

562   began when leaf water potentials declined below -3 MPa (Walker, 2014). Consequently, decline in

563   Ψ_{leaf} with decreasing turgor would not necessarily enhance water uptake into leaves as they dried.

564   Indeed, a low ε would allow the concentration of the cytoplasm to vary significantly over the range of

565   positive turgor, whereas a high ε would keep the cytoplasmic concentrations similar, and also reduce

566   the possibly deleterious effects of changes in cell geometry. Thus, we agree with the second

567   suggestion that a high modulus of elasticity coupled with a low solute potential enables leaves to

568   maintain the levels of hydration required for cellular function during drying to the turgor loss point

569   (Cheung *et al.*, 1975, Bartlett *et al.*, 2012).

570

571   The extent of water storage in domains 1 and 2 revealed by PV curve could play an important role in

572   the maintenance of diurnal photosynthetic activities in leaves of *A. marina*. Specifically, extracellular

573   water storage capacitance of domain 1 ($\mathbf{Q_x}$) was about five times greater than the intracellular water

574   storage capacitance of domain 2 ($\mathbf{Q_e}$). However, $\mathbf{Q_x}$ was functional over a range of Ψ_{leaf} approximately

575 five times less than that of Q_e. Consequently, extra and intracellular water storage was about equal. In

576 other words, storage of water in extracellular spaces doubled the amount of water available for use in

577 gas exchange without loss in turgor. For example, area-specific evaporation rates measured for *A.*

578 *marina* grown at the same location as those in the present study ranged from 1 to 2 mmol H_2O m^{-2} s^{-1}

579 (Martin *et al.*, 2010). So a leaf with extracellular water storage capacitance of 69 g H_2O m^{-2} MPa^{-1}

580 (Table 4) or 3800 mM H_2O m^{-2} MPa^{-1} as estimated from domain 1, when fully charged, could alone

581 supply the water loss needed to support photosynthesis at the above evaporation rates for 3230 to

582 1615 s, that is for 54 to 27 min with only 0.85 MPa decrease in Ψ_{leaf}. Once extracellular water is

583 exhausted, then the water storage capacitance of domain 2 (i.e. 13 g H_2O m^{-2} MPa^{-1} or 700 mM H_2O

584 m^{-2} MPa^{-1}) could alone support the same rates of gas exchange for another 50 to 25 min until the

585 turgor loss point (i.e. -5.1 MPa) is reached. Given the relative proportions of space occupied by

586 different tissue layers in the leaf lamina (Table 3), the hypodermis alone could have buffered water

587 loss from the mesophyll by contributing almost half of the water available for photosynthesis during

588 dehydration over domain 2 (all in the absence of water flux from the roots). Thus, water stored within

589 a leaf could supplement that provided by the roots, thereby extending the duration of gas exchange

590 activities for perhaps about two hours longer than otherwise, which could be critically important for a

591 species when soil salinity constrains the supply of water to the leaves.

592

603

604

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725

Table 1. Abbreviations

Parameter	Symbol	Unit
Calculation with extracellular water included	+	
Calculation without extracellular water	-	
Water mass difference	Δw	g
Leaf water potential gradient	$\Delta \Psi$	MPa
Leaf dry mass	DM	g
Leaf fresh mass	FM	g
Maximum leaf fresh mass	FM_{max}	g
Water storage capacitance	**Q**	g MP^{-1}m^{-2}
Water storage capacitance at domain 2	**Q$_e$**	g MP^{-1}m^{-2}
General water storage capacitance	**Q$_g$**	g MP^{-1}m^{-2}
Water storage capacitance at domain 1	**Q$_x$**	g MP^{-1}m^{-2}
Relative water content	RWC	%
RWC at turgor loss point	RWC_{π}^{0}	%
RWC at full turgor	RWC_{π}^{100}	%
RWC at the transition between domains 1 and 2	RWC_x	%
Relative water deficit	RWD	%
RWD at turgor loss point	RWD_{π}^{0}	%
RWD during domain 2	RWD_e	%
RWD during domain 1	RWDx	%
Leaf area	S	cm^2, m^2
Maximum leaf water content	WC_{max}	g
Extracellular water content	WC_x	g
Pressure gradient	ΔP	MPa
RWC difference	ΔRWC	%
Fractional decrease in a cell's water volume	$\Delta V/V$	
Volumetric modulus of elasticity	**ε**	MPa
Bulk modulus of elasticity at domain 2	**ε$_e$**	MPa
General bulk modulus of elasticity	**ε$_g$**	MPa
Bulk modulus of elasticity at domain 1	**ε$_x$**	MPa
Leaf water potential	Ψ_{leaf}	MPa
Leaf water potential at the transition between domains 1 and 2	Ψ_x	MPa
Leaf water potential at full hydration	Ψ_{100}	MPa
Leaf water potential at turgor loss point	Ψ_{π}^{0}	MPa
Osmotic potential at full turgor	Ψ_{π}^{100}	MPa

Table 2. Summary of PV curve components and their calculations as previously described (Scholander *et al.*, 1964, Turner, 1988, Tyree & Ewers, 1991, Bartlett *et al.*, 2012).

Parameter	Symbol	Unit	Calculation
Water storage capacitance	**Q**	g MP^{-1} m^{-2}	$Q = \dfrac{\Delta w}{\Delta \Psi}\dfrac{1}{S}$ $= \dfrac{\Delta RWC.\ WC_{max}}{\Delta \Psi}\dfrac{1}{S}$
Bulk modulus of elasticity	**ε**	MPa	$\varepsilon = \dfrac{\Delta P}{\frac{\Delta V}{V}} = \dfrac{\Delta \Psi}{\Delta RWC}$
Maximum water content	**WC$_{max}$**	g	$WC_{max} = FM_{max} - DM$
Relative water content	**RWC**	%	$RWC = (FM - DM)/WC_{max}$
Leaf water potential gradient	Δ**Ψ**	MPa	$\Delta \Psi = |\Psi_{tlp} - \Psi_{100}|$
Relative water content deficit	**RWD**	%	$RWD = 100 - RWC$

Table 3. Properties of fully expanded, field-grown, sun leaves of *A. marina* used for measurements of PV curves and anatomical analyses. Values are mean ± se (n = 5, except for * where n = 15)

Parameter		Symbol	Unit	Mean	se (n = 5)
Leaf area		**S**	cm^2	14.8	1.1
Maximum fresh mass		**FM$_{max}$**	g	1.04	0.07
Dry mass		**DM**	g	0.38	0.02
Maximum water content		**WC$_{max}$**	g	0.66	0.05
Leaf mass per area		**LMA**	g m^{-2}	256.3	13.9
Maximum water content per dry mass		**WC$_{max}$ DM^{-1}**	g g^{-1}	1.76	0.08
Leaf thickness			µm	588	10
Contribution to leaf thickness	Upper epidermis		%	3	0
	Hypodermis		%	36	1
	Palisade mesophyll		%	30	1
	Spongy mesophyll		%	13	0
	Trichome		%	19	1
Trichome	Density		cm^{-2}	218,758	8,783
	Volume*		µm^3	20,460	3,288 (n = 15)

Table 4. Leaf water relations of *A. marina* derived from three – domain PV curves as shown in Fig. 1. Values are mean ± se, n = 5. Note that symbols + or – denote whether extracellular water was included or excluded, respectively, in the calculations.

Parameter	Symbol	Unit	Mean	se (n = 5)
Relatively water deficit during domain 1	RWD_x+	%	13	1
Leaf water potential at RWC_x+	Ψ_x	MPa	-0.85	-0.03
Relative water deficit during domain 2	RWD_e+	%	12	1
Osmotic potential at full turgor	Ψ_π^{100}	MPa	-4.2	-0.1
Water potential at turgor loss point	Ψ_π^0	MPa	-5.1	-0.1
RWC at full turgor	RWC_π^{100}	%	87	1
RWC at turgor loss point, including extracellular water	RWC_π^0+	%	75	3
RWC at turgor loss point, excluding extracellular water	RWC_π^0-	%	86	2
General bulk modulus of elasticity	ε_g	MPa	21	2
Modulus of elasticity for domain 1	ε_e	MPa	7	1
Modulus of elasticity for domain 2	ε_x	MPa	37	4
General water capacitance	Q_g	g m^{-2} MPa^{-1}	22	2
Water storage capacitance for domain 1	Q_x	g m^{-2} MPa^{-1}	69	6
Water storage capacitance for domain 2	Q_e	g m^{-2} MPa^{-1}	13	2

Table 5. Composition of *A. marina* petiolar gel. Values are mean percent dry mass of the total dry mass of the polysaccharides named in the table below ± se, n = 3. Abbreviations: Rha: Rhamnose, Ara: Arabinose, Xyl: Xylose, Man: Mannose, ManUA: Mannuronic acid, Gal: Galactose, GalUA: Galacturonic acid, Glc: Glucose, GlcUA: Glucuronic acid. A: esterified uronic acid analysis, B: unesterified uronic acid analysis. NA: not applicable.

	Rha	Ara	Xyl	Man	ManUA	Gal	GalUA	Glc	GlcUA
A (%)	2.07	83.47	0.97	0.23	NA	10.60	NA	2.67	NA
se	0.19	0.32	0.09	0.03	NA	0.29	NA	0.22	NA
B (%)	2.23	83.17	0.80	0.23	0.00	10.53	0.00	0.53	2.50
se	0.19	0.50	0.06	0.03	NA	0.20	NA	0.15	0.15

Fig. 1. A representative pressure volume (PV) curve of an *Avicennia marina* leaf grown at Batemans Bay, New South Wales, Australia: (a) Relative water content, calculated with total leaf water content, (RWC+, %) as a function of leaf water potential in absolute value ($|\Psi_{leaf}|$, MPa). Key points and their x, y coordinates are indicated by open symbols. In domain 1, **A** *(open circle)* shows the point of full hydration at which $|\Psi_{leaf}|$ was 0.1 MPa and leaf RWC was set at 100%; the transition between domains 1 and 2 is indicated by **B** *(open diamond)*; **C** *(open triangle)* is the turgor loss point at the transition between domains 2 and 3. (b) Inverse leaf water potential in absolute value ($|1/\Psi_{leaf}|$, MPa^{-1}) as a function of relative water deficit, calculated with total leaf water content, (RWD+, %). The full data set is plotted in the inset graph, whereas points representing extracellular water (points **A** to **B** in (a)) were excluded from the data set plotted in the main graph. The main graph shows relationships among calculated values of Ψ_{leaf} at the turgor loss point (point **c,** open circle), the y intercept (**d$_0$**), and the intercept (**d$_x$**) at RWD+ = RWD$_x$+, i.e. relative water deficit during domain 1. At point **c**, $|1/\Psi_\pi^0| = 0.2$ so $|\Psi_\pi^0| = 5$ MPa. The linear regression between $|1/\Psi_{leaf}|$ and RWD+ after the turgor loss point was reached followed the equation: y = -0.0034x + 0.2682 (r² = 0.98). Therefore, $|1/\Psi_{leaf}|$ at **d$_0$** = 0.2682, and at **d$_x$** = 0.2376 as RWD+ at **d$_x$** = 11. Accordingly, $|\Psi_{leaf}|$ at **d$_0$** = 3.7 MPa and at **d$_x$** (Ψ_π^{100}) = 4.3 MPa.

Fig. 2. Transverse sections of (a) petiole, (b) leaf midvein, and (c) leaf lamina of *A. marina*. Symbols: B: bundle sheath extension; C: cisternae; F: fimbrial vein; H: hypodermis; P: palisade mesophyll; S: spongy mesophyll; T: trichomes; V: vascular bundle. Yellow arrows indicate mucus secretion glands in (a) and salt secretion glands in (b) and (c). Black arrow indicates stomate. Bars are 100 µm.

Fig. 3. Trichomes from the abaxial surface of *A. marina* leaves at different hydration states viewed with (a) bright-field microscopy and (b-e) cryo-SEM. (a) Trichome general structure. Symbols: ca: cap cell, st: stalk cell, ba: basal cell. (b) Both cap and stalk cells were filled with water at Ψ_{leaf} = -0.1 MPa. (c, d) Cap cells were collapsed but stalk cells maintained their shapes and were filled with gas at Ψ_{leaf} = -0.7 MPa. (e) The lower stalk cell maintained its shape while the cap and upper stalk cell were shrivelled and collapsed at Ψ_{leaf} = -4.3 MPa. Bars are 20 µm. (f) Diagrammatic representation of changes in trichomes with dehydration, showing a trichome fully expanded and filled with water (blue) at Ψ_{leaf} = -0.1 MPa, shrunken, filled with gas (white) and with a collapsed cap cell at Ψ_{leaf} = -0.25 MPa, and shrunken, filled with gas, and with the cap and upper stalk cells collapsed at Ψ_{leaf} = -4.3 MPa.

Fig. 4. Detection of Ψ_{leaf} threshold for draining of water-filled trichomes during dehydration in *A. marina* leaves. (a) Trichome layer covering abaxial leaf surface viewed under white light showing the cap cells (grey) and underline stalk cells (circular back area) of the trichomes and (b) under blue light showing the absence of detectable auto-fluorescence which could interfere with identification of the green fluorescence emitted by fluorescein under blue-exciting light. (c, d and movie 1 (Supplement S2)) The spread of fluorescein over the wet cap surface of water – filled trichomes (Ψ_{leaf} = -0.1 MPa). The images were collected at 10s (c) and 2 min (d) after fluorescein was applied to the cap surface at a distance approximately 1 mm from the observed area. Fluorescence intensity was initially weak (c) and increased with time (d) as more fluorescein diffused across the observed area. (e-f and movie 2 (Supplement S3)) Uptake of fluorescein applied to the cap surface of trichomes when Ψ_{leaf} = -0.25 MPa. The observed area partly included the place where fluorescein was applied. Fluorescence from the dye drop was saturating (white area in e), and reflected by surrounding trichomes (green area in e). The drop of fluorescein initially maintained its shape on the cap surface and then rapidly disappeared a minute later when the drop was absorbed by underlying trichomes (f). Note that some stalk cells were filled with dye (arrow in f) while in others, dye remained in the wall of cap and stalk cells and the surrounding area appeared black. Bars are 0.05 mm.

Fig. 5. Petiolar cisternae in leaves of *A. marina*. Distribution of cisternae (★★) in (a) transverse and (b) longitudinal sections as visualized by bright- field microscopy. Cryo-SEM micrographs of cisternae

filled with water when Ψ_{leaf} = -0.1 MPa (c), or air and droplets when Ψ_{leaf} = -0.1 MPa (c, d), Ψ_{leaf} = -0.7 MPa (e) and Ψ_{leaf} = -6 MPa (f). Bars are 50 μm in (a, b), and 25 μm in (c-f).

Fig. 6. Cryo-SEM micrographs of transverse sections through (a, b) pith parenchyma in the midvein and (c, d) paradermal sections through spongy mesophyll cells in the lamina of leaves of *A. marina* differing in leaf water status. Arrows point at extracellular spaces filled with ice, indicating the presence of liquid water (a, c) at Ψ_{leaf} = -0.06 MPa and with gas (b,d) at Ψ_{leaf} = -1 MPa. Bars equal 20 μm in (a, b) and 10 μm in (c, d).

Fig. 7. Plasmolysis in different cell types of leaves of *A. marina* after turgor had been lost (Ψ_{leaf} ≈ -6 MPa). (a) Petiolar collenchyma, (b) petiolar parenchyma surrounding gas-filled cisternae, (c) hypodermis, and (d) palisade mesophyll. Arrows indicate gaps between cell walls and cell membranes. Bars are 25 μm in (a, b), and 20 μm in (c, d).

Fig. 8. Diagrammatic summary of changes in the leaf lamina during dehydration through domains 1, 2, and 3 of the PV curve shown in Fig. 2. The major tissue layers of hypodermis (H), palisade (P), spongy mesophyll (S), and trichomes (T) are indicated as in Fig. 3. Salt secretion glands (purple) occurred on both leaf surfaces while stomata (pink dots) occurred only on the abaxial surface. At maximum hydration in Domain 1, all cells were turgid. Extracellular water (blue) occurred in a few extracellular spaces of the mesophyll where cells were in close apposition as in Fig. 6c. Trichomes were filled with water as in Fig. 4b, f. At the beginning of Domain 2, extracellular water was absent from spongy mesophyll as in Fig. 6d and trichomes as in Fig. 4c, d, f. Finally, after turgor was lost at -5 MPa in Domain 3, plasmolysis was evident with further dehydration to -6 MPa in most cell types, including hypodermis (Fig. 7c), palisade (Fig. 7d) and spongy mesophyll cells (data not shown). Plasmolysed cells are indicated by thin white gaps between cell walls and cell membranes. While the leaves declined in thickness during dehydration, cellular connections were maintained with no sign of cell collapse, except in the shrivelled, hollow cap and stalk cells of trichomes (Fig. 4e, f). The variation in panel size and colours indicate progressive shrinkage of the leaf lamina during dehydration from Domain 1 through Domain 3.

Supplement S1. Reanalysis of the data set from Fig.1, assuming domain 1 represented a "plateau effect". (a) Relative water content, calculated without extracellular water, (RWC-, %) as a function of leaf water potential in absolute value ($|\Psi_{leaf}|$, MPa) with the fully hydrated status (100% RWC) set at ***B** (open diamond).* Open dots indicate extracellular water (RWD_x-). (b) Inverse leaf water potential in absolute value ($|1/\Psi_{leaf}|$, MPa^{-1}) as a function of relative water deficit, calculated without extracellular water (RWD-,%). For reference, the full data set in Fig.1b, including the extracellular water, is plotted in the inset graph. Point **c (**open circle**)** is the turgor loss point and $|\Psi_\pi^0|$ = 5 MPa as in Fig. 1b. With the changes excluding extracellular water in RWD calculation, the equation for the linear regression between $|1/\Psi_{leaf}|$ and RWD- after **c** was reached was y = -0.003x + 0.2313 (r^2 = 0.98). Thus, the y intercept at point **d,** $|1/\Psi_\pi^{100}|$ = 0.2314 so $|\Psi_\pi^{100}|$ = 4.32 MPa.

Supplement S2: Movie showing diffusion of fluorescein across an abaxial leaf surface when Ψ_{leaf} = -0.1 MPa and trichomes were full of water (see Fig. 4 for further details).

Supplement S3: Movie showing absorption of fluorescein by draining trichomes when Ψ_{leaf} = - 0.25 MPa (see Fig. 4 for further details).