



**Daily irrigation attenuates xylem abscisic acid concentration and increases leaf water potential of *Pelargonium x hortorum* compared to infrequent irrigation**

Journal:	<i>Physiologia Plantarum</i>
Manuscript ID	PPL-2015-00299.R1
Manuscript Type:	Regular manuscript - Ecophysiology, stress and adaptation
Date Submitted by the Author:	17-Dec-2015
Complete List of Authors:	Boyle, Richard; Lancaster University, Lancaster Environment Centre McAinsh, Martin; Lancaster University, Lancaster Environment Centre Dodd, I.C.; Lancaster University, Lancaster Environment Centre
Key Words:	Abscisic acid, Irrigation frequency, Stomata, Water use efficiency, Leaf water potential

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3 **1 Daily irrigation attenuates xylem abscisic acid concentration and increases leaf water potential**  
4 **2 of *Pelargonium x hortorum* compared to infrequent irrigation**

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15 **7 Abstract**

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17 8 The physiological response of plants to different irrigation frequencies may affect plant growth and  
18 9 water use efficiency (WUE; defined as shoot biomass/cumulative irrigation). Glasshouse-grown,  
19 10 containerized *Pelargonium x hortorum* BullsEye plants were irrigated either daily at 100% of plant  
20 11 evapotranspiration (ET) (well-watered; WW), or at 50%ET applied either daily (FDI) or cumulatively  
21 12 every 4 days (IDI), for 24 days. Both FDI and IDI applied the same irrigation volume. Xylem sap was  
22 13 collected from the leaves, and  $g_s$  and  $\Psi_{leaf}$  measured every 2 days. As soil moisture decreased,  $g_s$   
23 14 decreased similarly under both FDI and IDI throughout the experiment.  $\Psi_{leaf}$  was maintained under  
24 15 IDI and increased under FDI. Leaf xylem ABA concentrations ( $[X-ABA]_{leaf}$ ) increased as soil  
25 16 moisture decreased under both IDI and FDI, and was strongly correlated with decreased  $g_s$ , but  $[X-$   
26 17  $ABA]_{leaf}$  was attenuated under FDI throughout the experiment (at the same level of soil moisture as  
27 18 IDI plants). These physiological changes corresponded with differences in plant production. Both FDI  
28 19 and IDI decreased growth compared to WW plants, and by the end of the experiment, FDI plants also  
29 20 had a greater shoot fresh weight (18%) than IDI plants. Although both IDI and FDI had higher WUE  
30 21 than WW plants during the first 10 days of the experiment (when biomass did not differ between  
31 22 treatments), the deficit irrigation treatments had lower WUE than WW plants in the latter stages when  
32 23 growth was limited. Thus, ABA-induced stomatal closure may not always translate to increased WUE  
33 24 (at the whole plant level) if vegetative growth shows a similar sensitivity to soil drying, and growers  
34 25 must adapt their irrigation scheduling according to crop requirements.

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46 **26 Abbreviations**

47 27  $\Psi_{leaf}$ , leaf water potential;  $\theta_{pot}$ , gravimetric soil water content;  $[X-ABA]_{leaf}$ , leaf xylem abscisic acid  
48 28 concentration; ABA, abscisic acid; ET, evapotranspiration; FDI, frequent deficit irrigation;  $g_s$ ,  
49 29 stomatal conductance; IDI, infrequent deficit irrigation; WUE, water use efficiency; WW, well-  
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### 33 Introduction

34 Changes in irrigation scheduling can be utilised to regulate crop growth and water use. Deficit  
35 irrigation applies water at a lower rate and/or volume than plant evapotranspirative (ET) demand, and  
36 offers an alternative to conventional irrigation management (that aims to full supply crop water  
37 requirements) (Álvarez et al., 2013). Whilst this may limit yield, this will also reduce the volume of  
38 water used (Geerts and Raes, 2009). In water limited situations, this can increase the water use  
39 efficiency (WUE) of the plant (Feres and Soriano, 2007). WUE may be defined at the whole plant  
40 level in terms of the harvestable yield (biomass of crop produced per unit of water used) (Blum,  
41 2005), or at the physiological level as the ratio of photosynthesis to transpiration (Hatfield et al.,  
42 2001). Ultimately the goal of many growers is to increase WUE. This may be achieved by using  
43 alternative deficit irrigation techniques that can be adapted to the needs to the grower (to either  
44 promote water savings and/or maintain plant quality).

45 Various adaptations of deficit irrigation have been studied, including “regulated deficit irrigation”  
46 (the application of deficit irrigation at specific stages of development during the growing cycle (Kang  
47 et al., 2000)) and “partial root zone drying” (lateral irrigation of half of the root zone, and withholding  
48 irrigation from the other half (Stoll et al., 2000)). Altering irrigation frequency is another promising  
49 approach that to date has received relatively little attention. This may present a viable option for  
50 growers to reduce irrigation inputs, and to maintain plant yield and quality. Manipulating irrigation  
51 volume and frequency (usually simultaneously) has been successful (in terms of water savings or  
52 increasing WUE) in arid or semi-arid regions where water is limited (El-Hendawy et al., 2008), or in  
53 golf course management (Fu and Dernoeden, 2009). These strategies involve delaying the application  
54 of water, typically in an attempt to reduce plant transpiration, limit excessive water losses by  
55 evaporation from the soil surface or through leaching below the crop rootzone, and to regulate water  
56 inputs in situations where growers over-irrigate plants (Wang et al., 2001). Since relatively few  
57 studies have investigated the effects of different irrigation frequencies applied at the same irrigation  
58 volume (but see Abalos et al., 2014, Ertek et al., 2004, Scagel et al, 2014), there is a limited  
59 understanding of the physiological impacts of irrigation frequency, which may limit accurate  
60 implementation of this technique.

61 An increase in the plant hormone abscisic acid (ABA) is a widely reported response to soil drying  
62 (Dodd, 2005). ABA is a potent anti-transpirant which can cause partial stomatal closure which may  
63 also limit carbon assimilation and thus growth (Medrano et al., 2002). However, the non-linear  
64 relationship between photosynthesis and stomatal conductance ( $g_s$ ) often results in partial stomatal  
65 closure which limits transpiration but maintains photosynthesis (and thus increases WUE) (Liu et al.,  
66 2006, Jones, 1992). In previous work examining the effect of different deficit irrigation frequencies  
67 (over a single drying cycle), leaf xylem ABA concentration ( $[X-ABA]_{\text{leaf}}$ ) of *Pelargonium x hortorum*

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3 68 increased as soil dried (and was correlated with decreasing  $g_s$  both *in vivo*, and in a transpiration  
4 69 bioassay where synthetic ABA was fed to detached leaves via the xylem), but  $[X-ABA]_{leaf}$  was  
5 70 attenuated when plants were irrigated daily at a fraction of crop ET, even at the same whole pot-soil  
6 71 moisture availability (Boyle et al., 2015). Since this may allow greater photosynthesis at the same  
7 72 level of soil moisture, identifying the mechanisms behind an attenuated  $[X-ABA]_{leaf}$  response is  
8 73 essential.

9 74 In *P. hortorum*, changes in  $[X-ABA]_{leaf}$  occurred independently of a consistent change in  $\Psi_{leaf}$   
10 75 (consistent with previous research (Sobeih et al., 2004)), suggesting that other mechanism(s) may act  
11 76 to regulate ABA levels (Boyle et al., 2015). Whilst it was concluded that this was likely a root-derived  
12 77 response (Boyle et al., 2015), it may also have been a temporal consequence of the different durations  
13 78 of irrigation treatment application (20 days of supplying irrigation at a fraction of crop ET vs 4 days  
14 79 of withholding irrigation). Further, attenuated ABA levels may be a result of “conditioning” due to  
15 80 both the duration and frequency of irrigation when plants were irrigated at a fraction of crop ET,  
16 81 priming the plant to respond with greater efficiency (Bruce et al., 2007, Goh et al., 2003). This  
17 82 response to altered irrigation frequency may have interesting implications for growers to increase  
18 83 WUE at the whole plant level, particularly if reduced  $[X-ABA]_{leaf}$  alters the balance between water  
19 84 loss and carbon gain by maintaining photosynthesis despite stomatal closure (Steuer et al., 1988).

20 85 The aim of this work was to determine the physiological (leaf water relations, stomatal conductance  
21 86 and xylem ABA concentration) and agronomic (growth and WUE) effects of irrigation frequency in  
22 87 plants exposed to long term deficit irrigation. We hypothesised that attenuated ABA signalling in  
23 88 response to frequent deficit irrigation could increase crop WUE, and investigated whether this  
24 89 response translated into physiological changes ( $g_s$  and  $\Psi_{leaf}$ ), as well as the effect on growth and  
25 90 WUE.

## 26 91 **Materials and Methods**

### 27 92 *Plant culture*

28 93 *Pelargonium x hortorum* BullsEye (zonal geranium) seeds were germinated in individual 13 cm x  
29 94 11.3 cm (1.05 L) pots (Pöppelman TEKU®, Germany) containing a peat based substrate (Levington  
30 95 M3) at a mean temperature of  $30 \pm 1^\circ\text{C}$  (daily maximum and minimum temperatures were  $37^\circ\text{C}$  and  
31 96  $16^\circ\text{C}$  respectively). Experiments were carried out in a naturally lit glasshouse. Supplementary lighting  
32 97 was provided when ambient PAR was less than  $500 \mu\text{mol m}^{-2} \text{s}^{-1}$  by high pressure sodium lamps  
33 98 (Osram Plantastar 600W) for a 14h photoperiod (0600 h-2000 h). A Hortimax growing solutions  
34 99 Ektron II (Pijnacker, The Netherlands) was used to record environmental conditions (air temperature  
35 100 and relative humidity) in the centre of the glasshouse.

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### 102 *Irrigation treatments*

103 Deficit irrigation treatments were modified from Boyle et al. (2015). Pots were weighed daily to  
104 calculate ET of all plants. Well-watered (WW) plants that received 100 % ET daily were used as a  
105 reference. To allow treatments to be applied over a longer duration, two groups of plants were subject  
106 to different irrigation regimes; infrequent (IDI; withholding water with regular re-watering events),  
107 and frequent deficit irrigation (FDI; daily irrigation at 50% of WW plants ET). After 4 days of  
108 withholding water, plants subject to IDI received the accumulated irrigation volume supplied to plants  
109 under FDI over the same cycle, which corresponded to approximately 63% of that supplied to WW  
110 plants. This ensured that both deficit irrigation treatments received the same volume of water during  
111 the treatment period (Fig. 1). Nutrients (Miracle-Gro All Purpose Soluble Plant Food, Miracle-Gro,  
112 Marysville, OH, USA) were applied at each IDI watering event, with all plants in each treatment  
113 receiving the same volume of nutrient solution over the duration of the experiment. Irrigation regimes  
114 were applied from 6 weeks after germination (when the canopy had covered the pot, thereby  
115 minimising evaporative losses from the soil) for 24 days, with six cycles of drying and re-watering  
116 (IDI) in total.

### 117 *Physiological measurements*

118 Stomatal conductance ( $g_s$ ) was measured using a porometer (Model AP4, Delta-T Devices,  
119 Cambridge, UK). Two readings of  $g_s$  were taken either side of the mid-rib on the youngest, fully  
120 expanded abaxial side of one leaf per plant. Leaf water potential ( $\Psi_{\text{leaf}}$ ) was measured immediately  
121 after measurements of  $g_s$  on the same leaf as described previously (Scholander et al., 1965), using a  
122 pressure chamber (Model 3000F01 Plant Water Status Console; Soil Moisture Equipment Corp. Santa  
123 Barbara, CA, USA). Detached leaves were transported in a sealed bag to the laboratory, and placed in  
124 the pressure chamber within 15 s of excision. Once in the chamber, the cut petiole was cleaned with  
125 deionised H<sub>2</sub>O and filter paper to remove cellular debris. Pressure was raised in the chamber at a rate  
126 of 0.02 MPa s<sup>-1</sup>, and  $\Psi_{\text{leaf}}$  was recorded when xylem sap collected on the surface of the cut petiole.  
127 After measuring  $\Psi_{\text{leaf}}$ , sap samples were stored for analysis of ABA concentrations by  
128 radioimmunoassay (Quarrie et al., 1988). Measurements of  $g_s$ ,  $\Psi_{\text{leaf}}$  and  $[X\text{-ABA}]_{\text{leaf}}$  were carried out  
129 every 2 days over the entire experimental period using different plants on each occasion.

### 130 *Plant harvest, water use efficiency and soil water status*

131 Plants were harvested every 2 days after physiological measurements. Shoot fresh weight, which was  
132 separated into leaves (excluding petioles) and stems, was measured. Leaf number was recorded, and  
133 leaf area was measured using a leaf area machine (LI-3100C Area Meter, LI-COR Inc., Lincoln, NE,  
134 USA). Root fresh weight was recorded on a separate group of plants three times during the  
135 experiment. Plant material was dried in an oven at 80°C until a constant mass to determine plant dry

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3 136 weight. Water use efficiency (WUE) was determined as the ratio of shoot dry weight and either  
4 137 irrigation volume (applied WUE) or plant ET (evapotranspirative WUE). After plants were harvested,  
5 138 the growth substrate (including plant roots) was weighed, dried in the oven for 7 days, and then  
6 139 reweighed to calculate gravimetric soil water content ( $\theta_{\text{pot}}$ ). A moisture release curve for this substrate  
7 140 (Dodd et al., 2010) allowed these measurements to be converted into soil matric potential.

#### 11 141 *Statistics*

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14 142 The six drying and re-wetting cycles were separated into two experimental phases, each comprising  
15 143 three drying and re-watering cycles to determine statistically whether the effects of the different  
16 144 treatments varied with experimental duration. The effect of irrigation treatment and irrigation phase  
17 145 on the relationship between plant and soil variables was tested using a three-way analysis of  
18 146 covariance (ANCOVA). Altered sensitivity of the  $y$ -variable to the  $x$ -variable is indicated by a  
19 147 significant interaction term. Differences between irrigation treatments, and treatments on each  
20 148 day/phase were evaluated by one-way analysis of variance (ANOVA) at  $p < 0.05$  using SPSS Statistics  
21 149 20 (IBM). When ANOVA was significant, means were discriminated using *Tukey's* multiple  
22 150 comparison test. Where values were not normally distributed according to a Shapiro-Wilk test, data  
23 151 was Log transformed and re-tested. If values were again found not to be normally distributed, a non-  
24 152 parametric Kruskal-Wallis test was used to determine if significant differences occurred between  
25 153 treatments and days. All graphs were created using Sigmaplot 8 (Systat Software Inc.).

#### 32 154 **Results**

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35 155 After 12 days of treatment, both IDI and FDI had decreased shoot fresh weight (by 30% and 26%  
36 156 respectively) compared to WW plants (Fig. 2a). After 24 days of treatment, IDI and FDI had  
37 157 decreased shoot fresh weight by 39% and 28% respectively, yet there was no significant difference in  
38 158 cumulative ET between these treatments (Fig. 2b). However, the temporal dynamics of ET varied,  
39 159 where IDI showed a series of declines and peaks in ET, which corresponded with soil drying followed  
40 160 by re-watering. Under IDI, recovery time of ET was similar in each cycle, generally increasing over  
41 161 48 h after re-watering, before declining over the subsequent 24-48 h. ET of WW plants steadily  
42 162 increased over the experimental period (Fig. 3a). FDI resulted in a more stable ET, which was  
43 163 typically lower than WW plants (by 28% averaged over the entire experiment).

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49 164 Irrigating plants under WW conditions maintained a relatively constant  $\theta_{\text{pot}}$  (Fig. 3b) averaging 3.0 g  
50 165  $\text{g}^{-1}$ , corresponding to a soil matric potential of -0.2 kPa. In contrast, both FDI and IDI showed a  
51 166 decrease in  $\theta_{\text{pot}}$  over the experimental period, with IDI showing periodic peaks of  $\theta_{\text{pot}}$  coinciding with  
52 167 each re-watering event. Minimal  $\theta_{\text{pot}}$  under FDI and IDI treatments of 1.2 and 0.6  $\text{g}^{-1}$  corresponded  
53 168 respectively to soil matric potentials of -40 kPa and less than -100 kPa respectively (Dodd et al.,  
54 169 2010).

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3 170 Over the entire experiment, stomatal conductance of WW plants averaged  $691 \pm 102 \text{ mmol m}^{-2} \text{ s}^{-1}$   
4 171 (Table. 1). Both FDI and IDI decreased  $g_s$  by approximately 87% and 91% respectively compared to  
5 172 WW plants. Stomatal conductance decreased similarly with decreasing soil moisture under both  
6 173 deficit irrigation treatments (Fig. 3c & 4a), which was consistent throughout the experiment (no-  
7 174 significant treatment  $\times$  phase  $\times$   $\theta_{\text{pot}}$  interaction; Table. 2)).

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11 175 Over the entire experiment,  $\Psi_{\text{leaf}}$  of WW plants averaged  $-0.8 \pm 0.1 \text{ MPa}$  (Table. 1). In contrast,  $\Psi_{\text{leaf}}$  of  
12 176 FDI plants gradually increased over the sampling period, whilst IDI plants showed a decrease in  $\Psi_{\text{leaf}}$ .  
13 177 There was no significant effect of the experimental phase on  $\Psi_{\text{leaf}}$  (Table. 2). There was no  
14 178 relationship between  $\Psi_{\text{leaf}}$  and  $\theta_{\text{pot}}$  under IDI (Fig. 4b & 5a), whilst under FDI,  $\Psi_{\text{leaf}}$  increased over the  
15 179 duration of the experiment (significant phase  $\times$   $\theta_{\text{pot}}$  interaction), and was thus correlated with  $g_s$  (Fig.  
16 180 4b & 5a).

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21 181 Over the entire experiment,  $[\text{X-ABA}]_{\text{leaf}}$  of WW plants averaged  $14 \pm 3 \text{ nM}$  (Table. 1). In contrast,  
22 182 both FDI and IDI increased  $[\text{X-ABA}]_{\text{leaf}}$ , with this increase being significantly higher under IDI  
23 183 (Table. 1). ABA increased as  $\theta_{\text{pot}}$  decreased under both deficit irrigation treatments independent of  
24 184 phase (no significant phase  $\times$   $\theta_{\text{pot}}$  interaction), although  $[\text{X-ABA}]_{\text{leaf}}$  was attenuated under FDI  
25 185 (significant treatment  $\times$   $\theta_{\text{pot}}$  interaction; Table. 3, Fig. 4c). Furthermore, there was no correlation  
26 186 between  $\Psi_{\text{leaf}}$  and  $[\text{X-ABA}]_{\text{leaf}}$  under either deficit irrigation treatment (Table. 3, Fig. 5b), whilst a  
27 187 consistent relationship between increased  $[\text{X-ABA}]_{\text{leaf}}$  and decreased  $g_s$  was observed under both FDI  
28 188 and IDI (no significant treatment  $\times$   $\theta_{\text{pot}}$  interaction; Table. 3, Fig. 6)

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34 189 Compared to WW plants, both deficit irrigation treatments decreased shoot dry weight and leaf area  
35 190 (Fig. 7c & Table. 1). Plant WUE was initially higher under both IDI and FDI treatments compared to  
36 191 WW plants, but in the latter stages of the experiment, WW plants had the highest WUE (Fig. 7a, b),  
37 192 especially when WUE was calculated per unit irrigation, rather than per unit evapotranspiration.

## 38 193 Discussion

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43 194 Decreasing irrigation frequency is a strategy that could potentially be implemented in nurseries as a  
44 195 water saving technique (Beeson, 2006). Although plants under FDI showed a more stable ET over the  
45 196 experimental period (albeit lower than WW plants) cumulative ET did not differ between IDI and FDI  
46 197 (Fig. 2b). Nevertheless, reducing irrigation frequency (IDI) increased ET after re-watering (typically  
47 198 within 48 h), then decreased ET over the subsequent 48 h (Fig. 3a). The peaks of ET under IDI  
48 199 suggest a rapid (1-2 days), partial recovery of leaf gas exchange upon re-watering (Fig. 3a). Thus  
49 200 altering the temporal dynamics of water use had no impact on total water use over the growing period.

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55 201 Stomatal closure of *P.hortorum* is a well characterised response to soil drying, which is tightly  
56 202 regulated to limit water loss (Álvarez et al., 2013, Sánchez-Blanco et al., 2009, Boyle et al., 2015). In  
57 203 the current study,  $g_s$  decreased similarly as soil moisture decreased under both deficit irrigation

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3 204 treatments (Table. 1; see also Boyle et al. (2015)), and this was sustained over the entire experimental  
4 205 period. Furthermore, irrigation treatment and experimental phase did not influence the relationship  
5 206 between  $g_s$  and  $\theta_{pot}$  (Fig. 4a). This supports current understanding that *P.hortorum* have particularly  
6 207 sensitive stomata (Sánchez-Blanco et al., 2009, Arora et al., 1998), in which  $g_s$  is tightly linked to soil  
7 208 moisture availability. This conserved response (independent of the frequency of soil drying) is  
8 209 evidently an important mechanism to prevent excessive water loss as soil moisture decreases, meriting  
9 210 further investigation to establish the physiological mechanism(s) involved.

14 211 Leaf water status can provide a valuable indicator of plant stress, as well as having a role in stomatal  
15 212 regulation (Buckley, 2005). Initially all treatments showed similar  $\Psi_{leaf}$  (Table. 1), and whilst in IDI  
16 213 plants  $\Psi_{leaf}$  later decreased compared to WW plants, there was ultimately no relationship between  $\Psi_{leaf}$   
17 214 and  $\theta_{pot}$  for IDI plants over the entire experiment (Fig. 4b). This was in contrast to FDI, where the  
18 215 slower imposition of soil drying, along with regular re-watering and decreased  $g_s$  acted to maintain a  
19 216 more positive  $\Psi_{leaf}$  (Table. 1, Fig. 4b) (Sperry et al., 2002). Thus the dynamics of soil drying and re-  
20 217 wetting altered  $\Psi_{leaf}$  at a given  $\theta_{pot}$ . Furthermore,  $\Psi_{leaf}$  was not related to  $g_s$  under either deficit  
21 218 irrigation treatment (Fig. 5a), supporting previous suggestions that it is not the key regulator of  $g_s$  in  
22 219 *P.hortorum* (Boyle et al., 2015). This is consistent with reports showing that *P.hortorum* has a low  
23 220 lethal  $\Psi_{leaf}$  threshold, which is either maintained (or improved) through particularly sensitive stomata,  
24 221 providing a regulatory mechanism for water loss (Augé et al., 2003).

31 222 The lack of a robust relationship between  $\Psi_{leaf}$  and  $g_s$  suggests that ABA may in fact be the central  
32 223 regulator of stomata in *P.hortorum* following long-term deficit irrigation treatments. Indeed, [X-  
33 224 ABA] $_{leaf}$  increased under both FDI and IDI as  $\theta_{pot}$  decreased (Fig. 4c), showed no relationship with  
34 225  $\Psi_{leaf}$  (Fig. 5b), and was strongly correlated with  $g_s$  (Fig. 6). However, [X-ABA] $_{leaf}$  was attenuated  
35 226 under FDI, even when *P.hortorum* plants were subject to the same irrigation treatment durations (Fig.  
36 227 4c). The similar sensitivity of  $g_s$  to [X-ABA] $_{leaf}$  in both deficit irrigation treatments contradicts  
37 228 previous reports that decreased  $\Psi_{leaf}$  sensitises stomata to ABA (Tardieu and Davies, 1992) since  $\Psi_{leaf}$   
38 229 was higher under FDI (Table. 1). Furthermore, this strengthens the argument (along with the findings  
39 230 of (Boyle et al., 2015)), that there is a limited role for other anti-transpirants regulating stomata in  
40 231 *P.hortorum* under soil drying.

47 232 The data presented here indicate that the attenuated ABA response previously observed (Boyle et al.,  
48 233 2015) was not due to different treatment duration (20 days for plants that were irrigated daily at a  
49 234 fraction of crop ET compared to withholding irrigation for 4 days). One possible explanation for the  
50 235 increased [X-ABA] $_{leaf}$  response in IDI plants may have been soil hysteresis (which can lower matric  
51 236 potential at a given soil water content) caused by regular drying and re-wetting of the substrate (Dodd  
52 237 et al., 2015). However, the consistent response of [X-ABA] $_{leaf}$  to  $\theta_{pot}$  (Fig. 4c) throughout the  
53 238 experiment suggests this is unlikely (although this response may differ in non-peat based substrates).



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3 239 Alternatively, it seems more likely this is a root derived response, which may have a number of  
4 240 potential explanations, including decreased synthesis of root ABA (Zhang and Tardieu, 1996),  
5 241 decreased flux of ABA from the roots (Jokhan et al., 1996), and variation in soil moisture distribution  
6 242 (Puertolas et al., 2013) between the different irrigation treatments. Future work should focus on  
7 243 understanding why frequent deficit irrigation attenuates the  $[X\text{-ABA}]_{\text{leaf}}$  response.

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11 244 Previous research reported that plant growth can be inhibited by increased ABA concentrations  
12 245 (Gowing et al., 1990) or decreased  $\Psi_{\text{leaf}}$  (Munns et al., 2000) decreasing cell wall extensibility (thus  
13 246 limiting leaf expansion), and/or stomatal limitation of photosynthesis (thus impairing biomass  
14 247 accumulation) (Medrano et al., 2002). Both FDI and IDI significantly decreased growth (biomass and  
15 248 leaf expansion) compared to WW plants over the entire experiment (Table. 1, Figs. 2a & 7c). Leaf  
16 249 expansion decreased concurrently with shoot biomass accumulation (Table. 1), so the general  
17 250 decrease in growth under the deficit irrigation treatments was likely due to stomatal closure (and thus  
18 251 impaired carbon assimilation (Medrano et al., 2002)). Since IDI and WW plants showed no  
19 252 pronounced differences in  $\Psi_{\text{leaf}}$ , and  $\Psi_{\text{leaf}}$  actually increased under FDI (Fig. 4b), it suggests that  
20 253 growth limitation is not primarily a hydraulic response. This is consistent with previous work  
21 254 demonstrating leaf growth inhibition without a decrease in  $\Psi_{\text{leaf}}$  (Martin-Vertedor and Dodd, 2011).  
22 255 While increased ABA concentrations (Creelman et al., 1990) may limit growth by decreasing cell  
23 256 wall extensibility (Van Volkenburgh and Davies, 1983), the similar leaf area of FDI and IDI plants  
24 257 (Table. 1), yet higher ABA concentrations in the latter (Fig. 4c), suggests that other phytohormonal  
25 258 changes may also be important in regulating growth (Albacete et al., 2008). Additional work should  
26 259 further investigate the mechanistic bases of this growth limitation as it may have important  
27 260 consequences for developing irrigation strategies to regulate canopy expansion of ornamental species.

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38 261 Two distinct phases of plant WUE were observed, consistent with the changes in biomass over the  
39 262 experimental period (Fig. 7). Initially, WUE increased under both deficit irrigation treatments, likely  
40 263 since stomatal closure limited water loss without limiting carbon assimilation (thus biomass growth  
41 264 was sustained). However, biomass accumulation was limited in the second phase of the experiment,  
42 265 likely due to a decrease in leaf expansion and photosynthesis as hitherto discussed. Clearly, sustained  
43 266 deficit irrigation (whether FDI or IDI) decreased plant growth, and the benefit of reduced water inputs  
44 267 was lost. This highlights that ABA-induced stomatal closure, at least over the longer term, does not  
45 268 always translate to increased WUE (at least at the whole plant level), which is an important  
46 269 consideration for growers scheduling their irrigation. This suggests that water-saving agriculture  
47 270 should focus less on saving water, and more on understanding the mechanisms that sustain biomass  
48 271 accumulation under reduced irrigation volumes.

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56 272 Previous studies investigating irrigation frequency on WUE have shown a varied response according  
57 273 to the species. When irrigation frequency (but not volume) was reduced (in soil-less culture), WUE

274 increased in cucumber when measured both with irrigation volume applied and plant  
275 evapotranspiration (Wang et al., 2009); had no effect on lettuce (Xu et al., 2004); and decreased in  
276 rose plants due to reduced biomass under infrequent irrigation (Katsoulas et al., 2006). However,  
277 WUE was not monitored frequently throughout these studies (as in Fig. 7), and there may be an  
278 optimal irrigation duration to increase WUE. For instance, the current study highlights that the initial  
279 period after treatments were imposed resulted in the highest WUE, whilst in pot grown teder plants  
280 subject to a single period of drying and re-watering, intrinsic WUE (leaf photosynthesis/ $g_s$ ) peaked  
281 during the drying phase in drought stressed plants then declined to control levels (Foster et al., 2015).  
282 Thus it may be necessary for growers to schedule their deficit irrigation at appropriate points of the  
283 cropping cycle to maximise WUE.

## 284 **Conclusions**

285 In summary, different deficit irrigation frequencies had different effects on the physiology, growth  
286 and WUE of containerised *P. hortorum*. Stomatal conductance decreased similarly under both deficit  
287 irrigation treatments, and was associated with increased  $\Psi_{\text{leaf}}$  under FDI. Rather,  $[X\text{-ABA}]_{\text{leaf}}$   
288 increased as soil moisture decreased, and was strongly correlated with decreased  $g_s$  under both deficit  
289 irrigation treatments. However,  $[X\text{-ABA}]_{\text{leaf}}$  was attenuated under FDI (at the same level of soil  
290 drying) when plants were exposed to different irrigation frequencies of the same duration. These  
291 physiological responses had longer term consequences on plant production, such that FDI plants had  
292 significantly higher shoot fresh weight compared to IDI plants by the end of the experiment. Although  
293 stomatal closure was sustained throughout the experiment, this does not always result in longer term  
294 increases in whole plant WUE (perhaps due to co-limitation of photosynthesis). Further studies  
295 measuring the impact of irrigation frequency on whole plant gas exchange are necessary (especially in  
296 the field) to reconcile ABA-mediated stomatal closure of individual leaves with whole plant responses  
297 that are important to irrigation practitioners.

## 298 **Acknowledgements**

299 This research was funded by the Centre for Global Eco-Innovation and the Agriculture and  
300 Horticulture Development Board (PO 017). The authors would like to thank Sarah Fairhurst of Arden  
301 Lea Ltd (Hesketh Bank, Preston, UK) for provision of seed and horticultural advice.

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Day 0	WW	3.1±0.0a	1011±129a	-0.70±0.02a	4±2a	306±14a	1.95±0.27a
	FDI	2.9±0.0a	990±87a	-0.68±0.02a	6±1a	322±41a	2.56±0.08a
	IDI	3.0±0.0a	1014±29a	-0.76±0.04a	2±1a	325±22a	2.23±0.40a
Phase 1	WW	3.2±0.0a	876±70a	-0.65±0.02a	5±1c	931±37a	4.14±0.44a
	FDI	1.3±0.2ab	127±13b	-0.67±0.05a	43±8b	706±31b	2.75±0.16b
	IDI	0.7±0.1b	53±11b	-1.00±0.03b	366±87a	680±45b	2.37±0.07b
Phase 2	WW	3.0±0.0a	276±66a	-0.73±0.07ab	13±9c	1523±37a	6.34±0.65a
	FDI	1.4±0.1b	65±25b	-0.55±0.03a	52±2b	1074±20b	4.02±0.27b
	IDI	1.3±0.1b	24±18b	-0.85±0.06b	177±80a	958±63b	3.41±0.32b

**Table 1.** Whole pot gravimetric water content ( $\theta_{\text{pot}}$ ;  $\text{g g}^{-1}$ ), stomatal conductance ( $g_s$ ;  $\text{mmol m}^{-2} \text{s}^{-1}$ ); leaf water potential ( $\Psi_{\text{leaf}}$ ; MPa); leaf xylem abscisic acid concentration ( $[\text{X-ABA}]_{\text{leaf}}$ ; nM); leaf area (LA;  $\text{cm}^2$ ); and root fresh weight (RFW; g) for *P. hortorum* plants subject to WW conditions, FDI or IDI (n=4). Data are means  $\pm$  SEM from Day 0, and the final days of sampling during Phases 1 and 2 (days 12 and 24 respectively). Different letters within a column and day/phase indicate significant differences according to a one-way ANOVA ( $p < 0.05$ ).

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**Table 2.** *P*-values from a three-way ANCOVA testing the interactive effects of applying either FDI or IDI to *P.hortorum* plants on stomatal conductance ( $g_s$ ) and leaf water potential ( $\Psi_{\text{leaf}}$ ), and the relationship between  $\Psi_{\text{leaf}}$  and  $g_s$ . Interactive effects were tested on data over two experimental phases, and from data where  $\theta_{\text{pot}}$  ranged between 1–3  $g\ g^{-1}$ .

	$\theta_{\text{pot}}$ vs $g_s$ <i>P</i> value	$\theta_{\text{pot}}$ vs $\Psi_{\text{leaf}}$ <i>P</i> value		$\Psi_{\text{leaf}}$ vs $g_s$ <i>P</i> value
Treatment	<b>0.051</b>	<b>&lt;0.001</b>	Treatment	<b>0.053</b>
$\theta_{\text{pot}}$	<b>&lt;0.001</b>	<b>0.001</b>	$\Psi_{\text{leaf}}$	<b>0.223</b>
Phase	<b>0.028</b>	<b>0.604</b>	Phase	<b>0.218</b>
Treatment* $\theta_{\text{pot}}$	<b>0.123</b>	<b>0.002</b>	Treatment* $\Psi_{\text{leaf}}$	<b>0.015</b>
Phase*Treatment	<b>0.270</b>	<b>0.028</b>	Phase*Treatment	<b>0.050</b>
Phase* $\theta_{\text{pot}}$	<b>0.579</b>	<b>0.304</b>	Phase* $\Psi_{\text{leaf}}$	<b>0.488</b>
Phase*Treatment* $\theta_{\text{pot}}$	<b>0.457</b>	<b>0.069</b>	Phase*Treatment* $\Psi_{\text{leaf}}$	<b>0.069</b>

**Table 3.** *P*-values from a three-way ANCOVA testing the interactive effects of applying either FDI or IDI to *P.hortorum* plants on abscisic acid (ABA), the relationship between ABA and  $g_s$ , and the relationship between  $\Psi_{\text{leaf}}$  and ABA. Interactive effects were tested on data over two experimental phases, and from data where  $\theta_{\text{pot}}$  ranged between 1–3  $g\ g^{-1}$ .

	$\theta_{\text{pot}}$ vs ABA <i>P</i> value		ABA vs $g_s$ <i>P</i> value		$\Psi_{\text{leaf}}$ vs ABA <i>P</i> value
Treatment	<b>0.003</b>	Treatment	<b>0.435</b>	Treatment	<b>0.868</b>
$\theta_{\text{pot}}$	<b>&lt;0.001</b>	ABA	<b>&lt;0.001</b>	$\Psi_{\text{leaf}}$	<b>0.176</b>
Phase	<b>0.066</b>	Phase	<b>&lt;0.001</b>	Phase	<b>0.829</b>
Treatment* $\theta_{\text{pot}}$	<b>0.020</b>	Treatment*ABA	<b>0.086</b>	Treatment* $\Psi_{\text{leaf}}$	<b>0.456</b>
Phase*Treatment	<b>0.952</b>	Phase*Treatment	<b>0.372</b>	Phase*Treatment	<b>0.241</b>
Phase* $\theta_{\text{pot}}$	<b>0.180</b>	Phase*ABA	<b>0.014</b>	Phase* $\Psi_{\text{leaf}}$	<b>0.960</b>
Phase*Treatment* $\theta_{\text{pot}}$	<b>0.796</b>	Phase*Treatment*ABA	<b>0.546</b>	Phase*Treatment* $\Psi_{\text{leaf}}$	<b>0.250</b>

**Figure 1.** Timetable of irrigation treatments, and the total volume of water applied to each treatment over the entire experimental period for *P.hortorum* plants subject to WW conditions, FDI or IDI. Black bar indicates WW irrigation, light gray bar indicates irrigation at 50 % ET daily (FDI), and dark gray indicates re-watering events when irrigation at 50 % ET every four days (IDI). Total water applied is mean data  $\pm$  SEM (n=13). Each phase is indicated by horizontal lines.

**Figure 2.** a) Shoot fresh weight (FW); b) cumulative evapotranspiration of *P.hortorum* plants subject to WW conditions, FDI or IDI. Bars represent means  $\pm$  SEM (n=4). Different letters indicate significant differences between irrigation treatments on each day according to a one-way ANOVA ( $p < 0.05$ ). Vertical lines indicate each re-watering event for the IDI treatment, and each phase is indicated by horizontal lines.

**Figure 3.** a) Evapotranspiration; b) Whole pot gravimetric water content; c) stomatal conductance ( $g_s$ ) of *P.hortorum* plants subject to WW conditions, FDI or IDI. Bars represent means  $\pm$  SEM (n=4). Different letters indicate significant differences between irrigation treatments on each day according to a one-way ANOVA ( $p < 0.05$ ). Vertical lines indicate each re-watering event for the IDI treatment, and each phase is indicated by horizontal lines.

**Figure 4.** a) Log stomatal conductance; b) leaf water potential; c) leaf xylem ABA concentrations of *P.hortorum* under different irrigation treatments over two experimental phases. Open symbols show data from plants subject to FDI (n=28); closed symbols show data from plants subject to IDI (n=28); half and half symbols shown data from WW plants (n=28). Data points are paired individual samples, regression line is fitted for data where  $\theta_{pot}$  ranged from 1-3  $g\ g^{-1}$ , and P values are reported.

**Figure 5.** a) Log stomatal conductance and b) leaf xylem abscisic acid concentration in response to leaf water potential of *P.hortorum* in drying soil under different irrigation treatments. Open symbols show data from plants subject to FDI (n=28); closed symbols show data from plants subject to IDI (n=28); half and half symbols shown data from WW plants (n=28). Data points are paired individual samples, regression line is fitted for data where  $\theta_{pot}$  ranged from 1-3  $g\ g^{-1}$ , and P values are reported.

**Figure 6.** Log stomatal conductance in response to leaf xylem abscisic acid concentration of *P.hortorum* in drying soil under different irrigation treatments. Open symbols show data from plants subject to FDI (n=28); closed symbols show data from plants subject to IDI (n=28); half and half symbols shown data from WW plants (n=28). Data points are paired individual samples, regression line is fitted for data where  $\theta_{pot}$  ranged from 1-3  $g\ g^{-1}$ , and P values are reported.

**Figure 7.** a) Applied water use efficiency; b) evapotranspirative water use efficiency; c) shoot dry weight; and d) total water applied (cumulatively until harvest) every two days of *P.hortorum* plants subject to WW conditions, FDI or IDI. Bars represent means  $\pm$  SEM (n=4). Different letters indicate



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3 significant differences between irrigation treatments on each day according to a one-way ANOVA  
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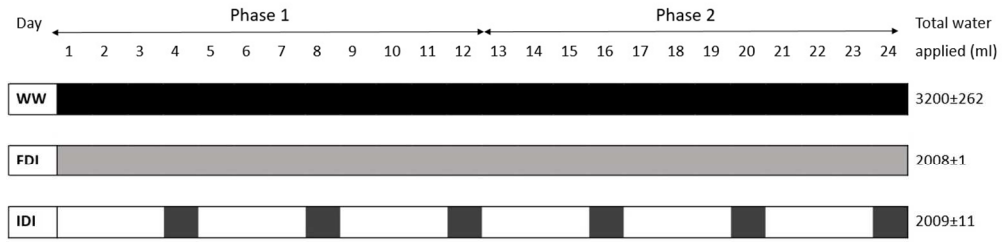


Figure. 1  
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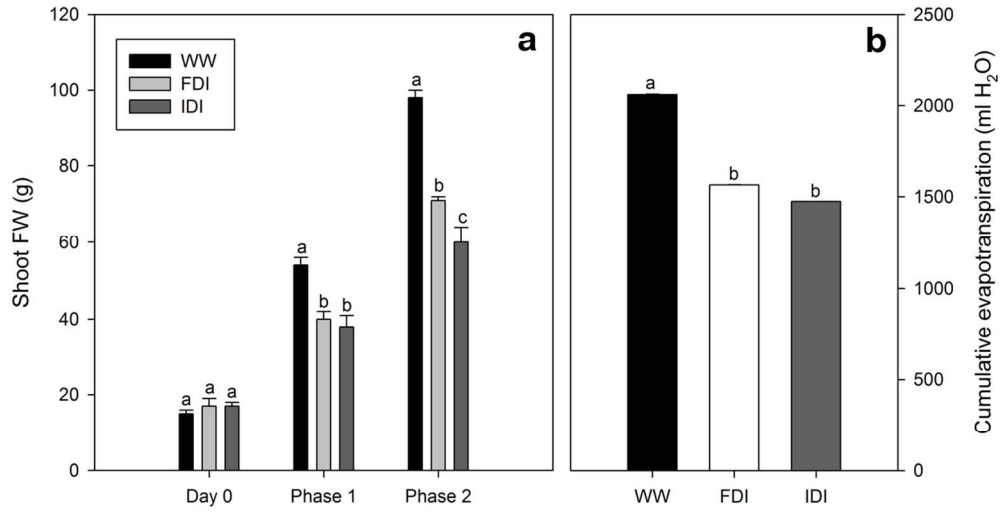


Figure.2  
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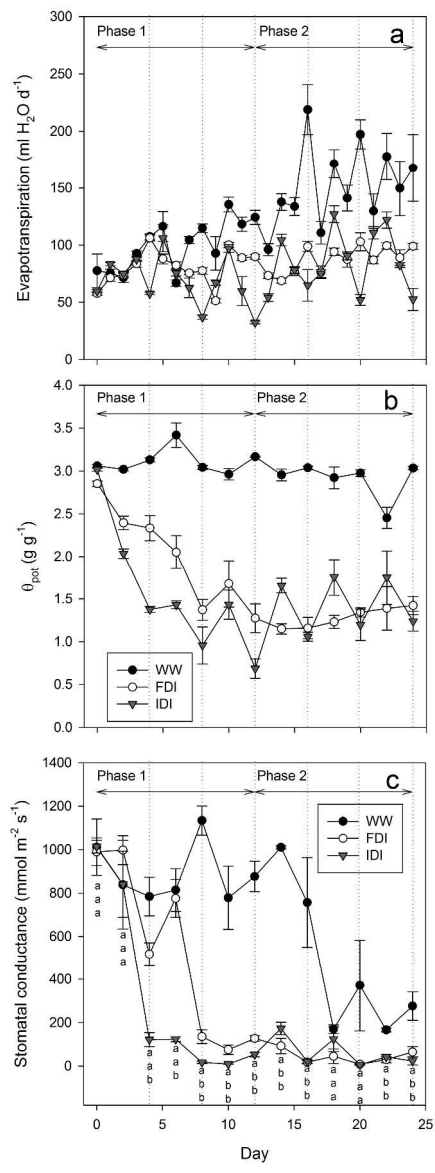


Figure. 3  
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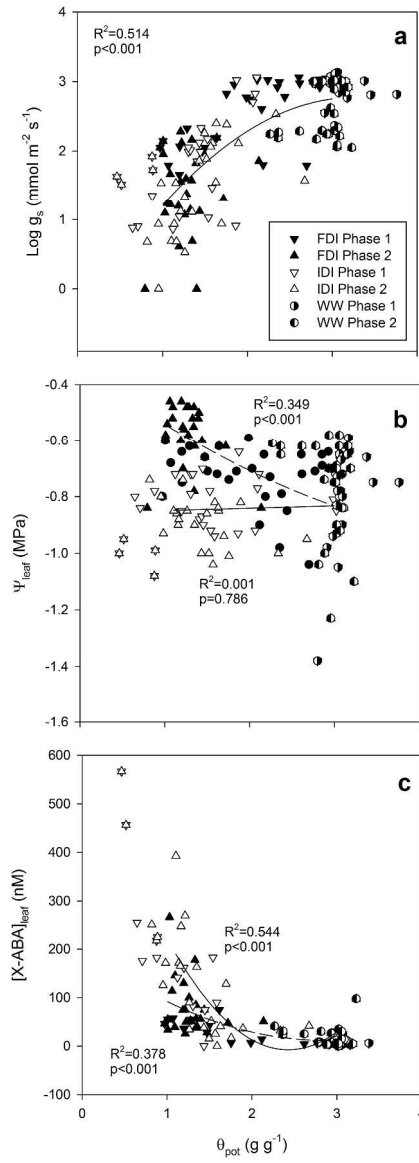


Figure. 4  
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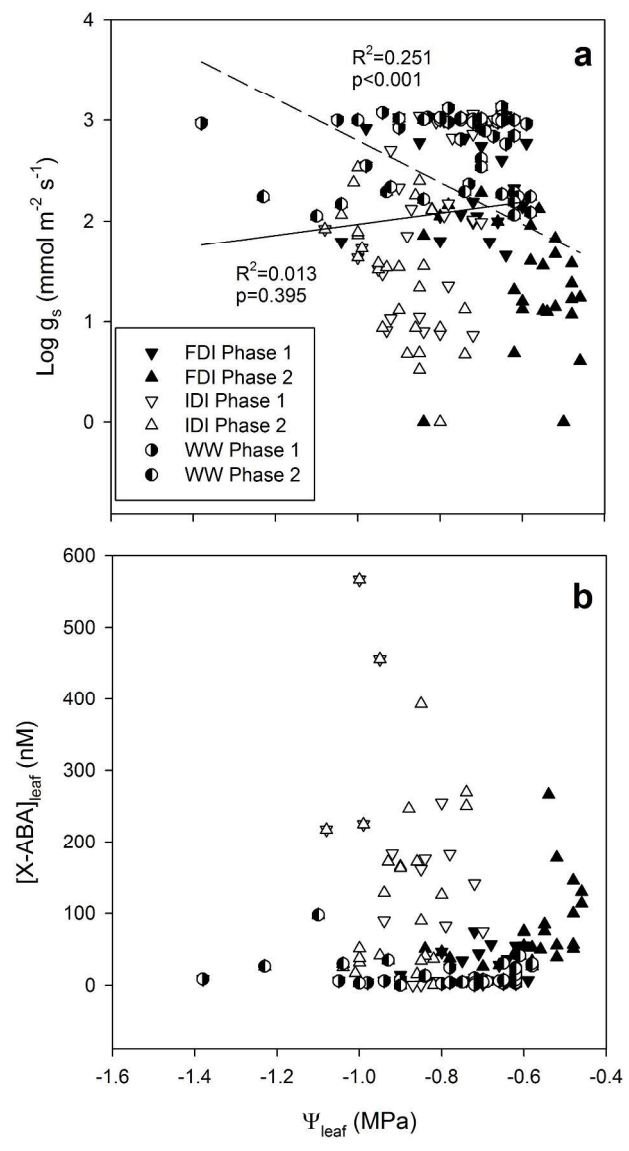


Figure. 5  
216x407mm (300 x 300 DPI)

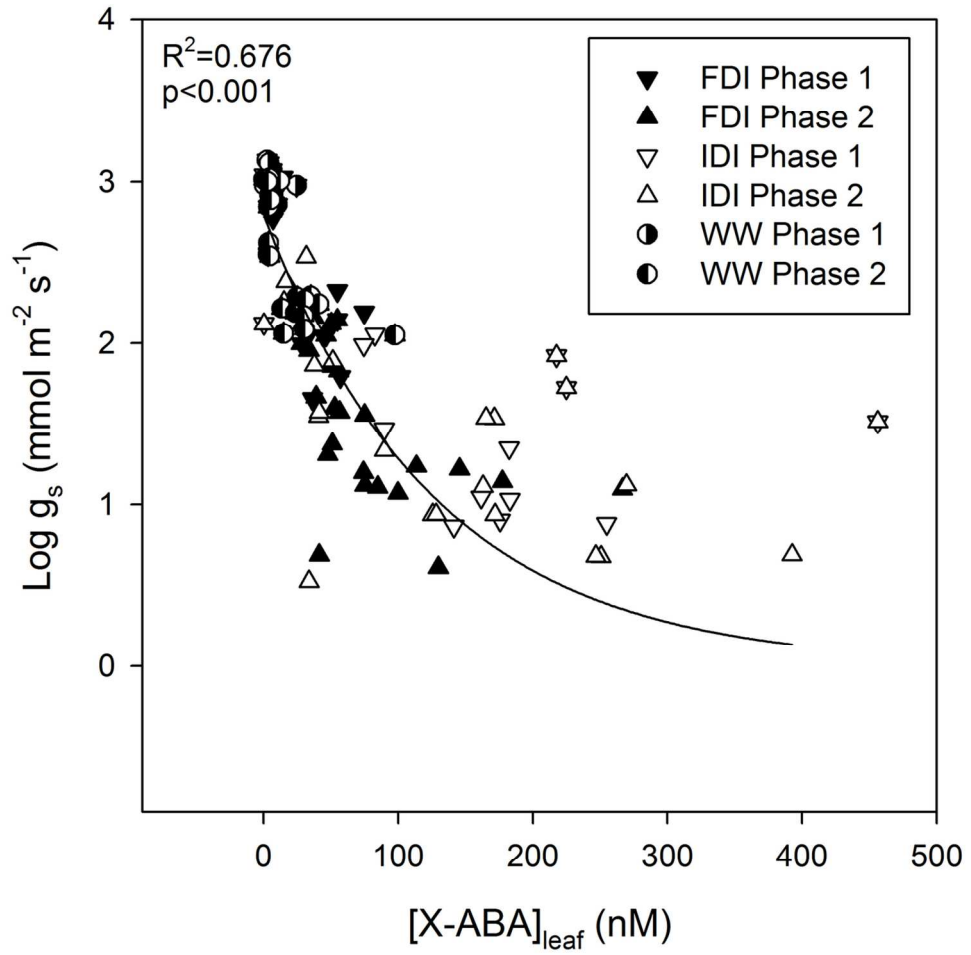


Figure. 6  
119x128mm (300 x 300 DPI)

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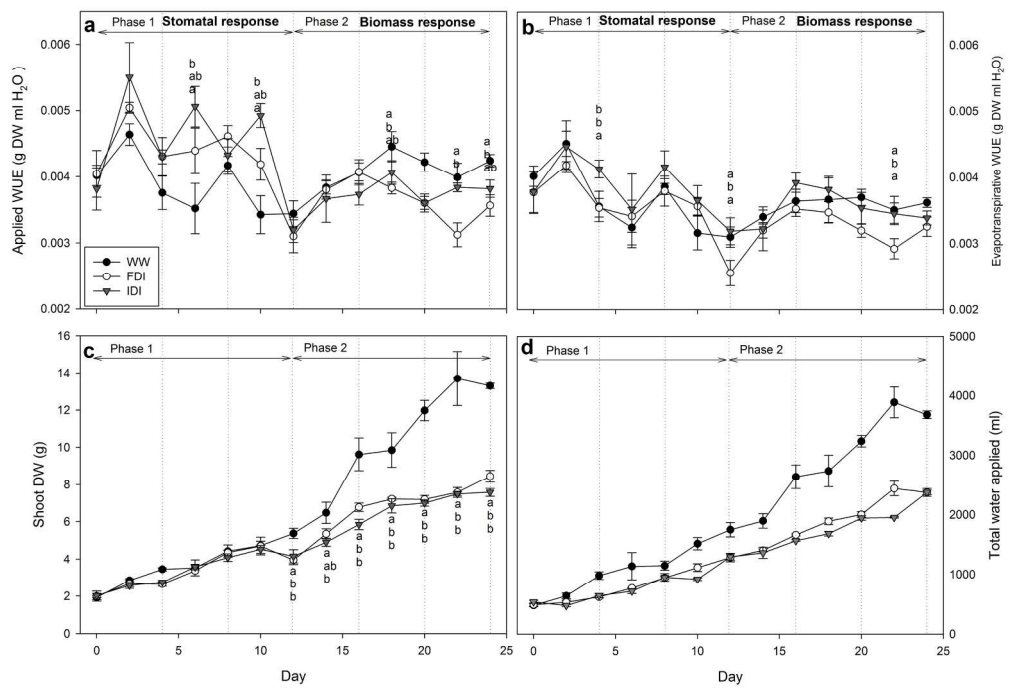


Figure. 7  
214x154mm (300 x 300 DPI)

Review