

1 **Response of stomatal density and bound gas exchange in leaves of maize to soil water deficit**

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23 **Abstract** Stomatal behavior in response to drought has been the focus of intensive research, but less
24 attention has been paid to stomatal density. In this study, 5-week-old maize seedlings were exposed to
25 different soil water contents. Stomatal density and size as well as leaf gas exchange were investigated
26 after 2-, 4- and 6-weeks of treatment, which corresponded to the jointing, trumpeting, and filling stages
27 of maize development. Results showed that new stomata were generated continually during leaf growth.
28 Reduced soil water content significantly stimulated stomatal generation, resulting in a significant
29 increase in stomatal density but a decrease in stomatal size and aperture. Independent of soil water
30 conditions, stomatal density and length in the trumpeting and filling stages were greater than in the
31 jointing stage. Irrespective of growth stage, severe water deficit significantly reduced stomatal
32 conductance (G_s), decreasing the leaf transpiration rate (T_r) and net photosynthetic rate (P_n). Stomatal
33 density was significantly negatively correlated with both P_n and T_r but more strongly with T_r , so the
34 leaf instantaneous water use efficiency (WUE_i) correlated positively with stomatal density. In
35 conclusion, drought led to a significant increase in stomatal density and a reduction in stomatal size and
36 aperture, resulting in decreased P_n and T_r . Because the negative correlation of stomatal density to T_r
37 was stronger than that to P_n , leaf WUE_i tended to increase.

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39 **Keywords** Drought • Stomatal density • Photosynthetic parameters • Maize

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45 **Introduction**

46 As a result of long-term evolution and adaptation to changing environmental conditions, astomatous
47 land plants with well-developed cuticles around their aerial organs declined in abundance, while
48 stomatous species predominated, and stomatal density appears to have increased monotonically
49 (McElwain and Chaloner 1995; Woodward 1998). Stomata, the small pores on the surfaces of leaves
50 and stalks that are bounded by a pair of guard cells, are the main portals of gas exchange between a
51 plant's above-ground organs and the atmosphere. The presence of stomata provides a means for
52 controlling diffusive water vapor loss from the leaf during transpiration and CO₂ entry into the leaf for
53 photosynthesis. Increasing the rate of transpiration is concomitant with improving the availability of
54 nutrients to the plant (Jones 1998). Total stomatal pore area may be only 5% of a leaf's surface, but the
55 rate of water vapor loss may reach 70% of that of a similar structure without a cuticle (Hetherington
56 and Woodward 2003). In terrestrial plants, only about 1–5% of root-absorbed water from soil is used
57 for structural composition and metabolism; the rest is lost into the atmosphere through transpiration
58 (Tesař et al. 2007). Thus, when soil water availability is limited, transpirative water loss through
59 stomata often can be the main factor limiting plant growth and development as well as crop yield.
60 Hence, decreasing transpirative water loss without impacting the growth and health is considered an
61 efficient pathway to increase water use efficiency of plants and reduce agriculture water use (Wang et
62 al. 2007).

63 Leaf transpirative water loss is controlled by stomatal development (including the size and density of
64 stomata on the epidermis) and behavior (stomatal aperture). Previous studies have reported the
65 responses of stomatal aperture to environmental factors such as light intensity, soil water availability,
66 the concentration of atmospheric CO₂, and endogenous plant hormones (Aminian et al. 2011; Busch

67 2014; Wang and Song 2008; Woodward et al. 2002). The molecular mechanism of stomatal movement
68 in response to environmental signaling has also been established, especially the abscisic-acid-mediated
69 signaling cascade in guard cells under drought (Hartung et al. 2002; Sauter et al. 2001; Schachtman and
70 Goodger 2008). In this response, plant species with larger stomata closes them more slowly, showing
71 lower drought sensitivity and, hence, a greater potential for hydraulic dysfunction. In contrast, small
72 stomata can open and close more rapidly and are generally associated with higher density, allowing for
73 rapid regulation of stomatal conductance (Aasamaa et al. 2001; Hetherington and Woodward 2003;
74 Royer 2001; Woodward et al. 2002). Hence, in drought environments, stomata are generally small
75 (Pearce et al. 2005; Sarker and Hara 2011; Spence et al. 1986), resulting in a decline in transpirative
76 water loss (Goodger et al. 2005; Yao 2001). However, this decrease in stomatal aperture may also
77 restrict photosynthetic CO₂ assimilation and, subsequently, plant growth and crop yield (Ripley et al.
78 2007).

79 While stomatal behavior has been the focus of intensive research, less attention has been paid to
80 stomatal density. Previous studies on the responses of stomatal density and leaf gas exchange to soil
81 drought also reported inconsistent results. Under drought stress, leaf stomatal density increased in
82 wheat (Quarrie and Jones 1977), *Populus trichocarpa* (Dunlap and Stettler 2001), olive (Bosabalidis
83 and Kofidis 2002), and *Solanum melongena* (Fu et al. 2013) but decreased in ginger (Xu et al. 2003)
84 and increased under moderate water deficit in *Leymus chinensis* but decreased under severe water
85 deficit (Xu and Zhou 2008). Differences in stomatal density further affect CO₂ and water vapor
86 exchanges between the leaf interior and the atmosphere. A recent study showed no correlations
87 between stomatal density and gas exchange parameters in *Arabidopsis* mutants with different stomatal
88 densities, and water stress could induce pore aperture to decrease but guard cell length to increase

89 (Lawson et al. 2014). Increased stomatal density in the *Arabidopsis* mutant *sdd1-1* also had no
90 significant influence on the net photosynthetic rate (P_n) (Schluter et al. 2003). However, Tanaka et al.
91 (2013) indicated that increased stomatal density increased CO₂ gas exchange and the photosynthesis
92 rate in *Arabidopsis thaliana*. Additionally, stomatal density was significantly positively correlated with
93 P_n , transpiration rate (T_r), and stomatal conductance (G_s) in *Leymus chinensis* (Xu and Zhou 2008), but
94 no or negative correlations between stomatal density and G_s were observed in Mediterranean plants and
95 wheat (Galmés et al. 2007; Wang et al. 2013). Hence, responses of stomatal size and density as well as
96 gas exchange to soil drought may depend on species and experimental conditions (Hetherington and
97 Woodward 2003; Wang et al. 2007). This limits the practical application of these experimental results
98 to a given crop species, especially for agricultural water conservation purposes. Also, little is known
99 about whether drought-induced variations in stomatal density or distribution are accurately correlated
100 to P_n or T_r .

101 The objectives of the present pot experiments were to (1) identify the generation period of new
102 stomata in maize growing without water limitation, (2) investigate the effects of different water
103 conditions on stomatal development and behavior as well as gas exchange, and (3) establish the
104 correlations between stomatal density and gas exchange in maize.

105

106 **Materials and methods**

107 Plant material and treatments

108 To understand the variations in stomatal number, density, and size during leaf development, seeds of a
109 common maize cultivar “Zhengdan 958” were sown individually in pots (10 cm high, 17 cm diameter,
110 ≈2.3 L volume) containing a mixture of humus and field soil (v/v = 1:1). After germinating, plants were

111 cultured in an artificial climate chamber at 25°C, a light intensity of 2 000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and a
112 photoperiod of 14h day/10h night under well-watered conditions. Every day, maize leaves of different
113 developmental ages (days) were excised ($n = 6$) then immediately observed and photographed using an
114 optical microscope (BX51, Olympus, Tokyo, Japan). Stomatal number, density, and size in the
115 microphotographs were analyzed with ImageJ 1.0 image processing software (National Institutes of
116 Health, Bethesda, MD, USA). Stomatal length and width (guard cells plus subsidiary cells) were
117 measured microscopically ($n = 40$). Stomatal number and density of leaves at different developmental
118 ages were calculated from 10 microscopic fields using stomatal counts and leaf area.

119 To investigate effects of soil water content on stomatal development and behavior, seeds of the
120 cultivar “Zhengdan 958” were sown individually in pots (30 cm high, 28 cm diameter, ≈ 18.5 L volume)
121 containing a mixture of local loess and vermiculite ($v/v = 1:1$). After germinating, plants were well
122 watered using Hoagland solution twice. Five weeks after sowing, plants were exposed to three soil
123 water contents: not water limited (corresponding to soil water content of $75 \pm 5\%$ field water capacity),
124 medium water deficit ($60 \pm 5\%$), and severe water deficit ($45 \pm 5\%$). Pots were weighed every day at
125 dusk to determine transpirative water loss, and soil water contents were maintained by compensating
126 transpirational water loss by adding tap water to the initial weight. Increasing plant weight was also
127 considered. Two, four, and six weeks after water treatments corresponded to the jointing (period of
128 rapid stem elongation), trumpeting (when tassels are visible but before silk emerges), and filling (kernel
129 formation) phases of maize growth. These three stages are critical periods of water demand for maize.
130 Stomatal density, size, aperture, and leaf gas exchange parameters were measured on the second
131 fully-expanded leaf from the top on six individual plants per treatment per stage.

132

133 Stomatal density, size, and aperture under different soil water contents
134 Stomatal density, size, and aperture in the center of the lower epidermis of the second fully-expanded
135 leaf from the top of plants in each treatment were observed and photographed using an optical
136 microscope at 100× ($n = 10$ microscopic fields), 400× ($n = 20$ stomata) and 1000× ($n = 40$ stomata)
137 magnification, respectively. The images were analyzed with ImageJ 1.0.

138

139 Measurement of leaf gas exchange

140 On cloudless days during the jointing, trumpeting, and filling stages, diurnal variations in leaf P_n , G_s ,
141 and T_r were measured from 07:00 to 19:00 at intervals of 2 hours using a LI-COR 6400XT portable
142 photosynthesis system (Li-COR Biosciences, Lincoln, NE, USA). The measurements were conducted
143 on the second fully-expanded leaf from the tops of six individual plants per each treatment. Leaf
144 instantaneous water use efficiency (WUE_i) was calculated using instantaneous values of P_n divided by
145 T_r .

146 A response curve of P_n to photosynthetically active radiation (PAR) was measured using an artificial
147 light source built into the LI-COR 6400 at radiation intensities of 0, 20, 50, 100, 200, 500, 800, 1 000, 1
148 200, 1 500, 2 000, and 2 500 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The light response curve was then plotted. Based on the
149 response curve of P_n as a function of PAR, a non-rectangular hyperbola model was fitted using the
150 statistics program SPSS 17.0 for Windows (IBM, Chicago, IL, USA) (Cannell and Thornley 1998),
151 where k is the curvature of the light-photosynthesis relationship:

$$152 \quad P_n = \frac{\text{PAR} \times \text{AQY} + A_{\max} - \sqrt{(\text{PAR} \times \text{AQY} + A_{\max})^2 - 4\text{AQY} \times \text{PAR} \times A_{\max}}}{2k} - R_d.$$

153 Maximum net assimilation rate (A_{\max}), dark respiration rate (R_d), apparent quantum yield (AQY)
154 were calculated from this response function. The light compensation point (LCP) and light saturation

155 point (LSP) were estimated using a regression equation for light and P_n in the radiation intensity range
156 from 0 to 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at $P_n = 0$ and $P_n = A_{\text{max}}$, respectively (Liu et al. 2005).

157

158 Statistical analysis

159 Significant differences among soil water treatments were analyzed with one-way ANOVA using SPSS

160 17.0. Differences were considered significant at $P < 0.05$. Correlations between parameters were
161 determined using linear regression.

162

163 **Results**

164 Stomatal development of maize leaves under well-watered conditions

165 The density and number of stomata were very low on newly-emerged leaves but increased significantly
166 in the early phase of leaf development (Fig. 1a), indicating that new stomata were generated during leaf
167 growth. When leaf area reached about 10 cm^2 , stomatal density stabilized at a relatively high level,
168 whereas the stomatal number increased continually as leaves grew. Stomatal length and width did not
169 change notably during leaf development (Fig. 1b).

170

171 Effects of soil water content on stomatal development of maize leaves in different growth stages

172 For fully-expanded mature leaves growing under well-watered conditions, stomatal density in the
173 trumpeting and filling stages were higher than that in the jointing stage, but the difference was not
174 statistically significant (Fig. 2a). In plants exposed to drought, stomatal density in each developmental
175 stage increased significantly, and the effects were enhanced with the increasing of drought.

176 Under the not-limited and medium drought conditions, stomatal length in the trumpeting and filling

177 stages were significantly greater than that in the jointing stage, while under severe drought stomatal
178 length in the later developmental stages also tended to increase (Fig. 2b). However, stomatal width
179 showed no significant difference among the three stages or water conditions (Fig. 2c). Irrespective of
180 the developmental stage, severe drought led to significant decreases in both stomatal length and width,
181 while the negative effects of medium drought were not statistically significant. The ratio of stomatal
182 length to width tended to increase under the three soil water contents and stages (Fig. 2d).

183

184 Effects of soil water content on stomatal aperture in different growth phases

185 Independent of soil water contents, leaf stomatal aperture did not significantly differ among
186 developmental stages. However, irrespective of developmental stage, both medium and severe drought
187 led to significant decreases in stomatal aperture compared with well-watered plants (Fig. 3).

188

189 Effects of soil water content on photosynthetic parameters in different growth stages

190 The diurnal patterns of T_r , G_s , and P_n were generally not changed by soil water content in the different
191 developmental stages (Fig. 4). T_r increased rapidly from 07:00 to a maximum at 11:00, remained
192 relatively high until 15:00, and then decreased quickly (Fig. 4a-c). Drought significantly decreased T_r
193 in the jointing stage only at 13:00 (Fig. 4a), while in the trumpeting and filling stages, significant
194 decreases occurred from 11:00-15:00 and 09:00-15:00, respectively, under severe drought but not
195 medium drought (Fig. 4b,c).

196 In each developmental stage, G_s was low in the early morning, reached a maximum at 11:00, and
197 then decreased gradually (Fig. 4d-f). Soil water deficit decreased G_s in the jointing and trumpeting
198 stages (Fig. 4d,e), but not in the filling stage (Fig. 4f). G_s in the filling and trumpeting stages were

199 generally higher than in the jointing stage.

200 P_n increased gradually from 07:00 to a maximum at 11:00, remained relatively stable level until
201 15:00, and then decreased gradually, irrespective of developmental stage or soil water content (Fig.
202 4g-i). Severe drought significantly decreased P_n in the jointing and filling stages, while the effects of
203 drought were not significant in the trumpeting stage.

204

205 Effects of soil water content on P_n response to PAR in different growth phases

206 Under different soil water contents and in different developmental stages, the response curves of P_n to
207 PAR showed similar trends: P_n increased rapidly with increasing PAR when $PAR < 500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$
208 and then remained relatively high (Fig. 5). In the jointing stage, severe drought, but not medium
209 drought, led to a significant decrease in the response of P_n to PAR when $PAR > 500 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. In
210 the trumpeting stage, plants under medium drought showed a significant increase in the response of P_n
211 to PAR, while the response was unaffected under severe drought. Soil water content did not have a
212 significant effect on the response of P_n to PAR in the filling stage.

213 Simulation using a non-rectangular hyperbola model showed that, in the jointing stage, medium
214 drought did not affect A_{max} and LSP but significantly reduced LCP, significantly increasing the range of
215 PAR (the difference between LSP and LCP), and AQY also significantly increased. However, severe
216 drought significantly reduced A_{max} and AQY while increasing LCP, leading to a significant decrease in
217 the range of PAR. In the trumpeting stage, medium drought significantly increased A_{max} , LCP, and R_d ,
218 whereas severe drought had no significant effect on these parameters. In the filling stage, increasing
219 drought significantly decreased A_{max} and LSP but increased LCP and R_d , resulting in a significant
220 decrease in the range of PAR (Table 1).

221

222 Correlations between stomatal density and G_s , P_n , T_r , and WUE_i

223 To further explore the effects of changes in stomatal density on leaf gas exchange, the correlations
224 between stomatal density and G_s , P_n , and T_r were analyzed. There was no correlation between stomatal
225 density and G_s (Fig. 6a). However, significantly negative correlations of stomatal density with P_n (Fig.
226 6b) and T_r (Fig. 6c) demonstrated that higher stomatal density reduced leaf P_n and T_r . Additionally, the
227 negative correlation of stomatal density with T_r was greater than that with P_n . Hence, leaf WUE_i was
228 positively correlated with stomatal density (Fig. 6d).

229

230 Correlations of G_s with P_n and T_r

231 The correlations of G_s to P_n and T_r in all of three developmental stages were similar. P_n and T_r increased
232 when G_s increased, and the trend was not significantly affected by water soil content (Fig. 7).

233

234 **Discussion**

235 Stomata are the portals of gas exchange between the interior of plant and the atmosphere; they control
236 CO_2 entry into the leaf for photosynthesis and diffusive water vapor loss from leaves during
237 transpiration. Their performance depends on their development and behavior, which are affected by
238 environmental factors (Hetherington and Woodward 2003; Wang and Song 2008). In recent decades,
239 the effects of drought on stomatal aperture have been extensively studied, and the mechanism of
240 drought-induced reduction in stomatal aperture or closure is well established. However, the responses
241 of stomatal density and related leaf gas exchange to drought have received less attention.

242 The results of this study showed that maize stomata were continually generated during leaf

243 expansion and growth, as reflected in increased stomatal number and a relatively-stable density, while
244 stomatal size (length and width) remained essentially unchanged. These phenomena are similar to
245 previous observations on tomato (Gay and Hurd 1975), *Amaranthus tricolor* (Ren 2004), and *Sorbus*
246 (Čaňová et al. 2012). Irrespective of developmental stage, severe drought, but not medium drought,
247 significantly increased stomatal density and significantly reduced stomatal size in maize, consistent
248 with numerous previous studies (Bosabalidis and Kofidis 2002; Dunlap and Stettler 2001; Fu et al.
249 2013; Pearce et al. 2005), but contradictory to the results of Xu et al. (2003) and Xu and Zhou (2008).
250 Small stomata could maintain the pores opening with lower guard-cell turgor pressures compared with
251 larger stomata (Spence et al. 1986). Hence, higher stomatal density and reduced stomatal size in maize
252 responding to drought can effectively inhibit transpirative water loss and better ensure water balance
253 (Bosabalidis and Kofidis 2002).

254 In previous studies, the correlations of stomatal density to P_n and T_r were different. Increased
255 stomatal density in wheat under drought was associated with reduced P_n and T_r (Wang et al. 2013) but
256 significantly positively correlated with P_n and T_r in *Leymus chinensis* exposed to moderate drought (Xu
257 and Zhou 2008). In the present study, the response of stomatal density to water deficit was independent
258 of growth stage. Increased stomatal density and an associated decrease in stomatal size under severe
259 drought correlated with a reduction in P_n and T_r but the negative association between stomatal density
260 and T_r was stronger than that with P_n , so leaf WUE_i trended to increase. Higher WUE_i is beneficial to
261 plant growth and development in severe drought conditions. Although G_s may be not always parallel
262 changes in leaf photosynthetic capacity (Caemmerer et al. 2004), results of this study showed
263 significantly positive correlations of G_s with P_n and T_r , similar to studies on bean (Lizana et al. 2006)
264 and pepper (Amor et al. 2010).

265 In conclusion, decreasing soil water content increased stomatal density but decreased stomatal size
266 and aperture, accompanied by a reduction in P_n and T_r . Independent of soil water conditions, stomatal
267 density and length in the trumpeting and filling stages were greater than that in the jointing stage.
268 Irrespective of growth stage, severe water deficit significantly reduced stomatal aperture and leaf G_s in
269 maize, consequently decreasing T_r and P_n . Stomatal density was more negatively correlated with T_r
270 than with P_n , so leaf WUE_i tended to increase.

271

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376 **Table 1** Simulated results of maximum net assimilation rate (A_{\max}), light compensation point (LCP),
 377 light saturation point (LSP), dark respiration rate (R_d), and apparent quantum yield (AQY) of maize
 378 leaves under different soil water conditions and in different growth phases (mean \pm SE, $n = 3$).

Treatment	A_{\max} ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	LCP ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	LSP ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	R_d ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)	AQY ($\mu\text{mol}\cdot\mu\text{mol}^{-1}$)
Jointing stage					
75%	41.02 \pm 2.56b	60.00 \pm 3.75b	2080 \pm 129a	2.97 \pm 0.19ab	0.05 \pm 0.003b
60%	41.53 \pm 2.59b	45.00 \pm 2.81a	2185 \pm 136a	2.61 \pm 0.16a	0.06 \pm 0.004c
45%	29.22 \pm 1.82a	80.00 \pm 5.00c	2025 \pm 126a	3.28 \pm 0.20b	0.04 \pm 0.003a
Trumpeting stage					
75%	33.31 \pm 3.21a	40.00 \pm 3.86a	1835 \pm 176a	2.42 \pm 0.23a	0.06 \pm 0.005ab
60%	41.59 \pm 4.01b	50.00 \pm 4.82b	1820 \pm 175a	3.03 \pm 0.29b	0.06 \pm 0.006b
45%	31.23 \pm 3.01a	40.00 \pm 3.86a	1765 \pm 170a	2.25 \pm 0.22a	0.06 \pm 0.005a
Filling stage					
75%	22.66 \pm 1.42b	30.00 \pm 1.87a	1360 \pm 84b	1.04 \pm 0.06a	0.04 \pm 0.002a
60%	20.98 \pm 1.31b	35.00 \pm 2.19b	1360 \pm 84b	1.57 \pm 0.10b	0.05 \pm 0.003c
45%	18.95 \pm 1.18a	45.00 \pm 2.81c	1160 \pm 72a	1.81 \pm 0.11c	0.04 \pm 0.003b

379 Different letters denote significant differences among soil water treatments at $P < 0.05$

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391 **Figure captions**

392 **Fig. 1** Variations in stomatal density and number (a) and size (length and width of guard cells plus
393 subsidiary cells) (b) during development of maize leaves under well-watered conditions.

394

395 **Fig. 2** Effects of soil water contents on stomatal density (a), stomatal length (b), stomatal width (c), and
396 the ratio of stomatal length and width (d) in different growth stages of maize. Different letters on error
397 bars indicate significant differences at $P < 0.05$

398

399 **Fig. 3** Effects of soil water content on stomatal aperture in different growth stages of maize. Different
400 letters on error bars indicate significant differences at $P < 0.05$

401

402 **Fig. 4** Effects of soil water content on diurnal variations in transpiration rate (T_r), stomatal conductance
403 (G_s), and net photosynthetic rate (P_n) in different growth stages of maize (mean \pm SE, $n = 6$)

404

405 **Fig. 5** Effects of soil water content on net photosynthetic rate (P_n) response to photosynthetically active
406 radiation (PAR) in different growth stages of maize (mean \pm SE, $n = 3$)

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408 **Fig. 6** Correlations of stomatal density with stomatal conductance (G_s) (a), net photosynthetic rate (P_n)
409 (b), transpiration rate (T_r) (c), and instantaneous water use efficiency (WUE_i) (d). Correlations between
410 parameters were determined using linear regression

411

412 **Fig. 7** Correlations of stomatal conductance (G_s) with net photosynthetic rate (P_n) and transpiration rate

413 (T_r) in different growth stages of maize. Correlations between parameters were determined using linear

414 regression

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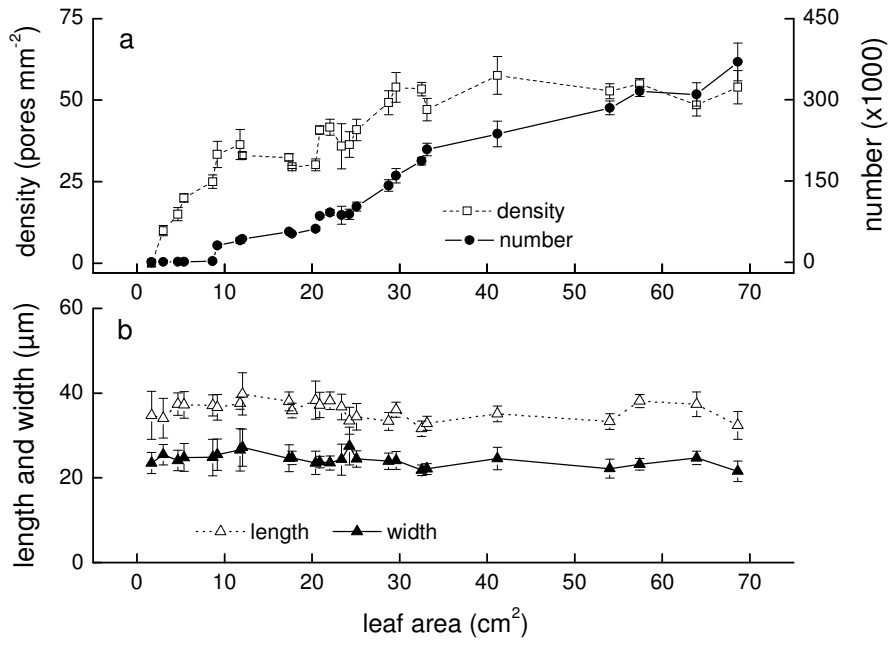
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435 **Fig. 1**



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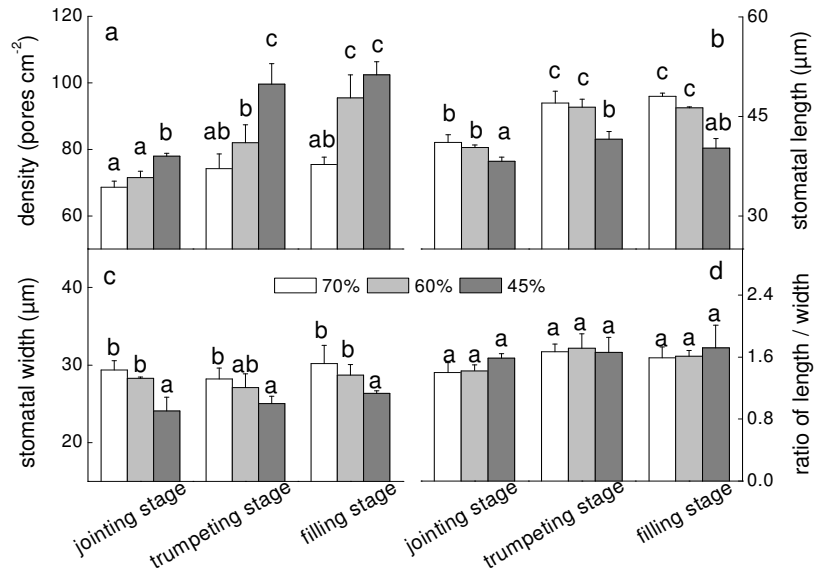
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450 **Fig. 2**



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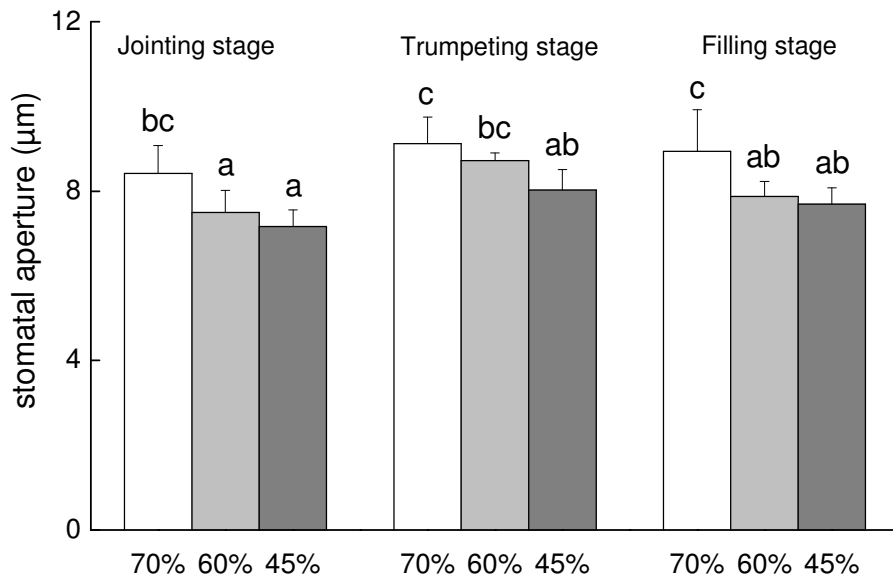
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465 Fig. 3



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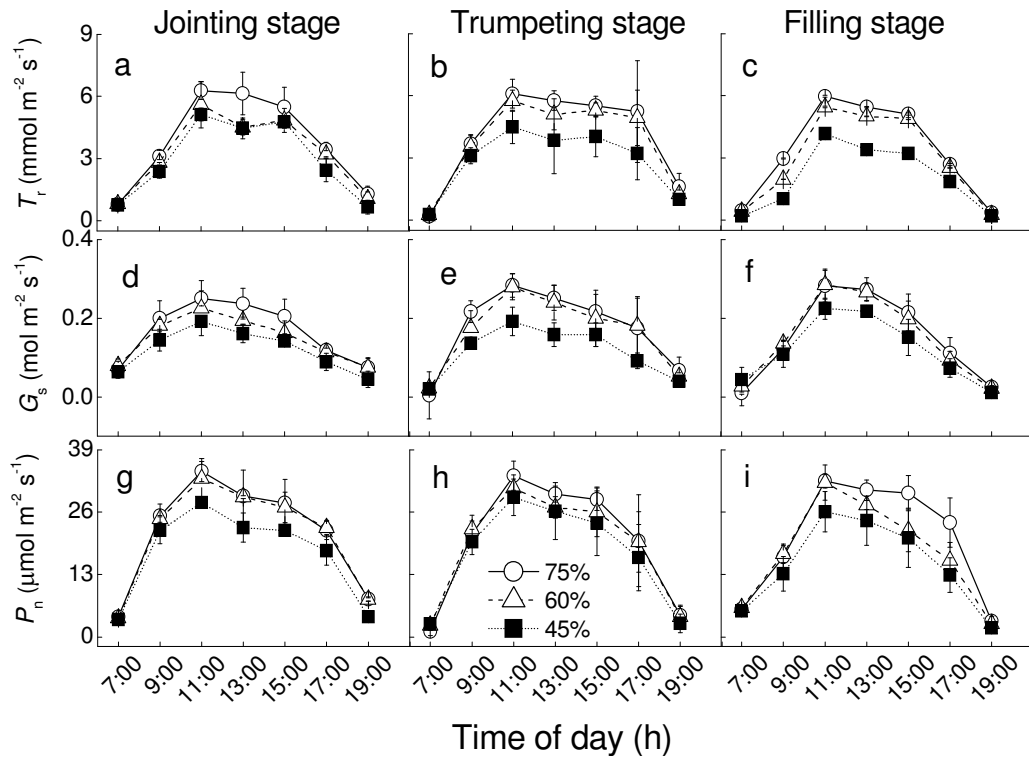
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480 Fig. 4



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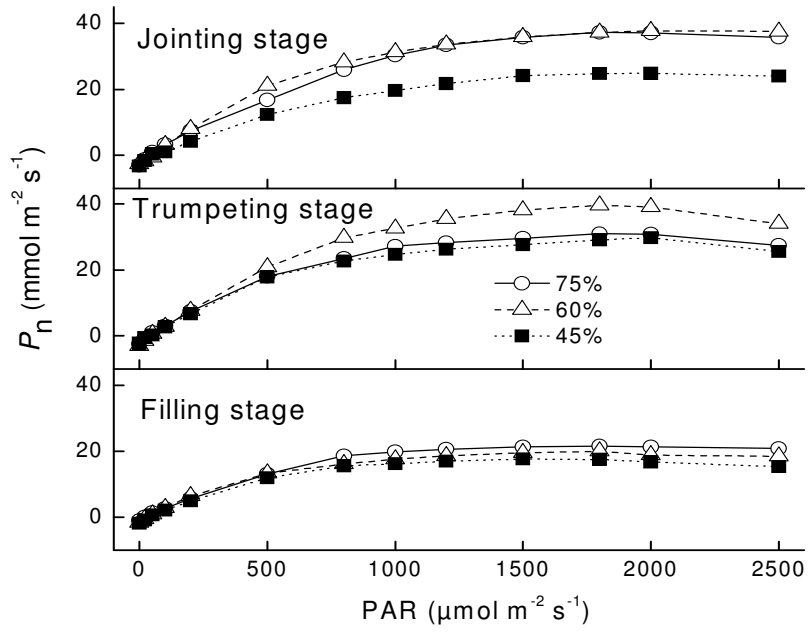
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494 Fig. 5



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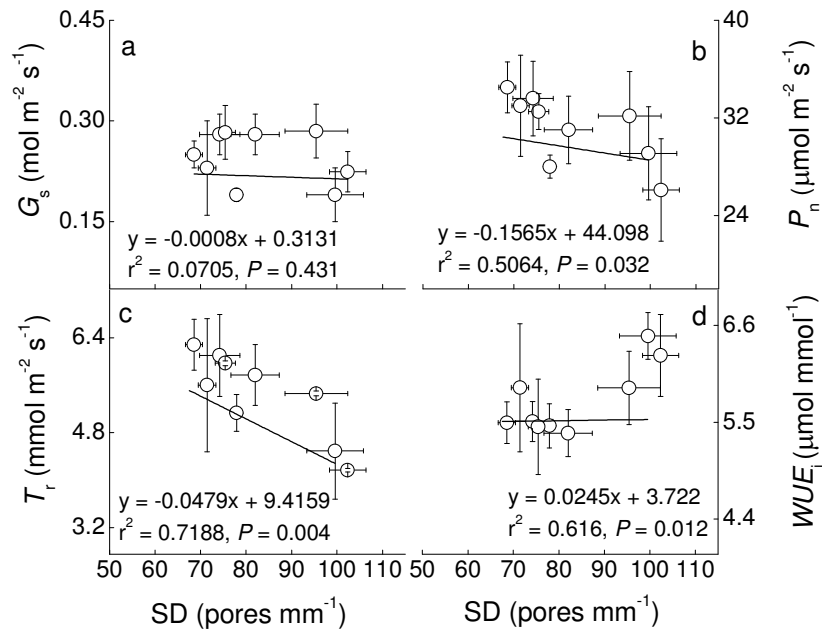
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509 **Fig. 6**



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