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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Introduction

As a result of long-term evolution and adaptation to changing environmental conditions, astomatous land plants with well-developed cuticles around their aerial organs declined in abundance, while stomatous species predominated, and stomatal density appears to have increased monotonically (McElwain and Chaloner 1995; Woodward 1998). Stomata, the small pores on the surfaces of leaves and stalks that are bounded by a pair of guard cells, are the main portals of gas exchange between a plant's above-ground organs and the atmosphere. The presence of stomata provides a means for controlling diffusive water vapor loss from the leaf during transpiration and CO_2 entry into the leaf for photosynthesis. Increasing the rate of transpiration is concomitant with improving the availability of nutrients to the plant (Jones 1998). Total stomatal pore area may be only 5% of a leaf's surface, but the rate of water vapor loss may reach 70% of that of a similar structure without a cuticle (Hetherington and Woodward 2003). In terrestrial plants, only about 1–5% of root-absorbed water from soil is used for structural composition and metabolism; the rest is lost into the atmosphere through transpiration (Tesař et al. 2007). Thus, when soil water availability is limited, transpirative water loss through stomata often can be the main factor limiting plant growth and development as well as crop yield. Hence, decreasing transpirative water loss without impacting the growth and health is considered an efficient pathway to increase water use efficiency of plants and reduce agriculture water use (Wang et al. 2007).

Leaf transpirative water loss is controlled by stomatal development (including the size and density of stomata on the epidermis) and behavior (stomatal aperture). Previous studies have reported the responses of stomatal aperture to environmental factors such as light intensity, soil water availability, the concentration of atmospheric CO_2, 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 CO_2 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 CO_2 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

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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 CO_2 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 μmol·m^{-2}·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 ± 5% field water capacity),

124   medium water deficit (60 ± 5%), and severe water deficit (45 ± 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.

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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.

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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 500μmol·m^{-2}·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:

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

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 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at $P_n = 0$ and $P_n = A_{max}$, respectively (Liu et al. 2005).

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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.

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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).

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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).

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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).

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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.

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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 μmol·m^{-2}·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 μmol·m^{-2}·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).

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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

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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).

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

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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 ± SE, $n = 3$).

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

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

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Figure captions

Fig. 1 Variations in stomatal density and number (a) and size (length and width of guard cells plus

subsidiary cells) (b) during development of maize leaves under well-watered conditions.

Fig. 2 Effects of soil water contents on stomatal density (a), stomatal length (b), stomatal width (c), and

the ratio of stomatal length and width (d) in different growth stages of maize. Different letters on error

bars indicate significant differences at $P < 0.05$

Fig. 3 Effects of soil water content on stomatal aperture in different growth stages of maize. Different

letters on error bars indicate significant differences at $P < 0.05$

Fig. 4 Effects of soil water content on diurnal variations in transpiration rate (T_r), stomatal conductance

(G_s), and net photosynthetic rate (P_n) in different growth stages of maize (mean ± SE, $n = 6$)

Fig. 5 Effects of soil water content on net photosynthetic rate (P_n) response to photosynthetically active

radiation (PAR) in different growth stages of maize (mean ± SE, $n = 3$)

Fig. 6 Correlations of stomatal density with stomatal conductance (G_s) (a), net photosynthetic rate (P_n)

(b), transpiration rate (T_r) (c), and instantaneous water use efficiency (WUE_i) (d). Correlations between

parameters were determined using linear regression

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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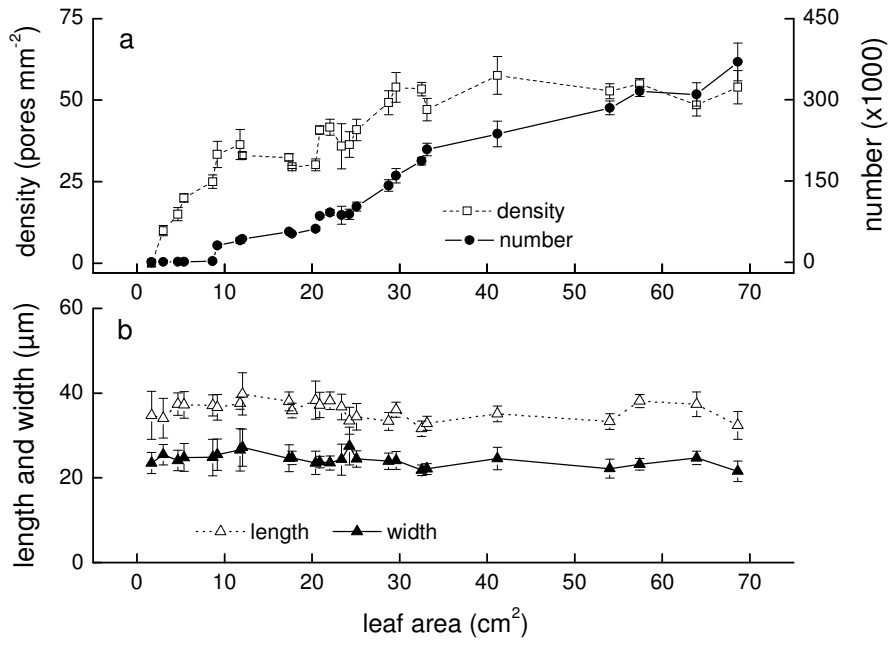
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435 **Fig. 1**

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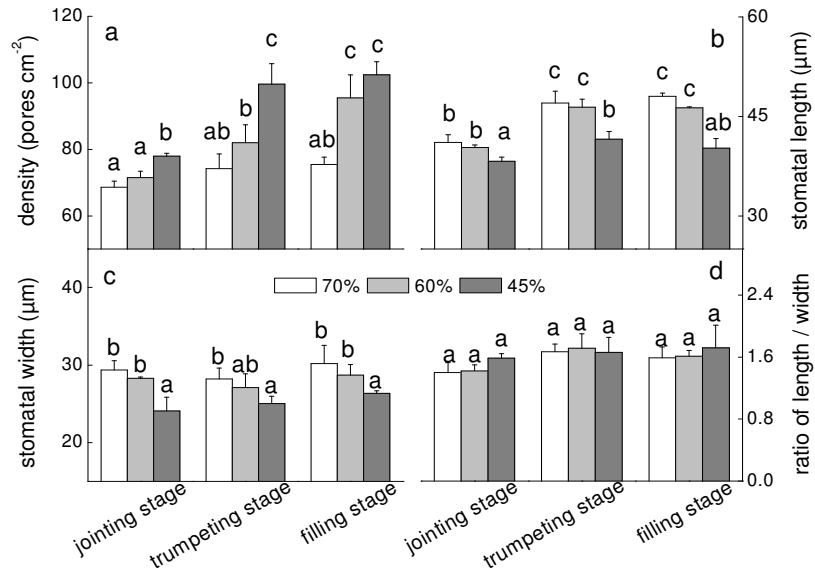437

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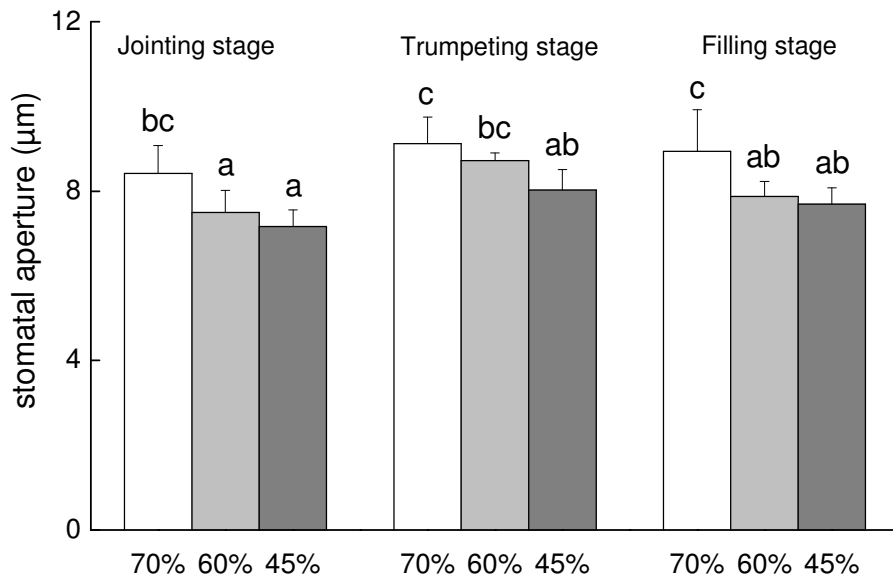
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450 **Fig. 2**

465 **Fig. 3**

Fig. 4
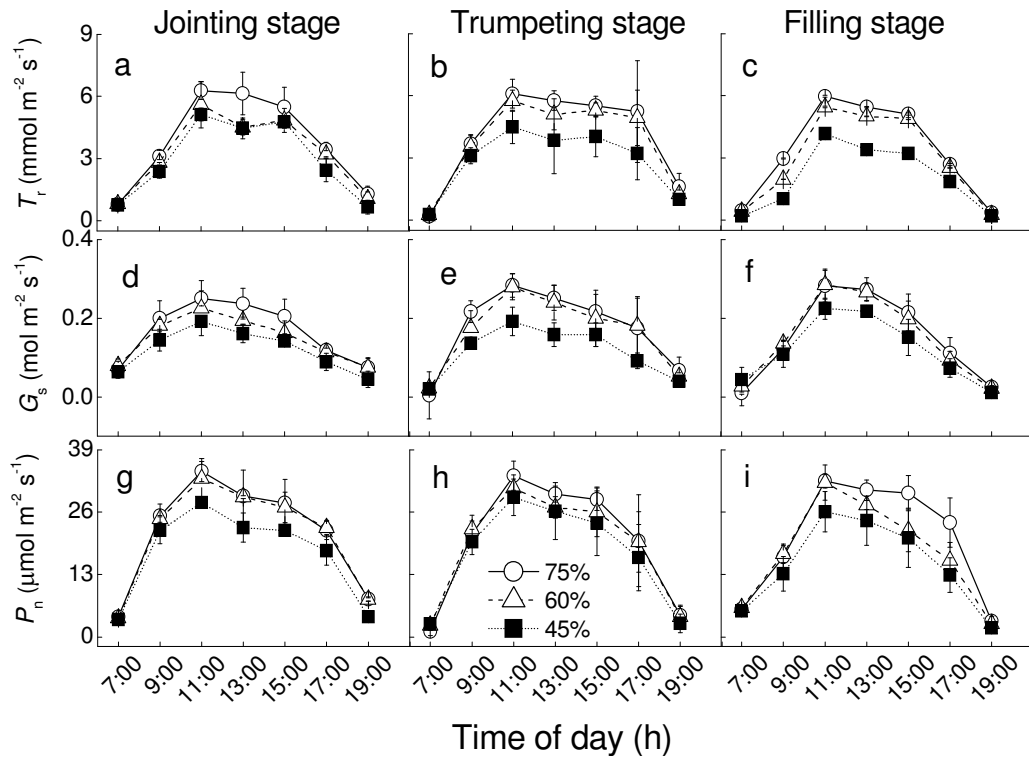
494   **Fig. 5**
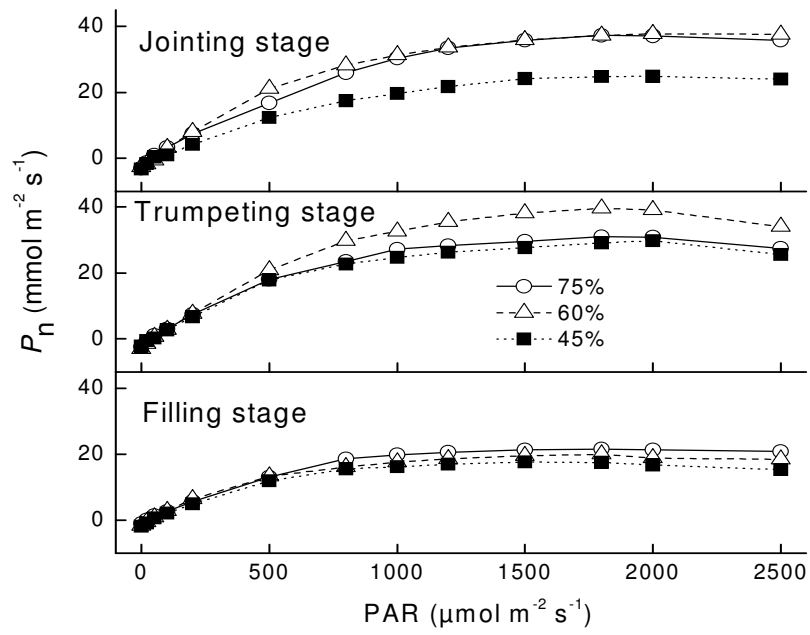
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509 **Fig. 6**

510 SD (pores mm⁻¹)   SD (pores mm⁻¹)
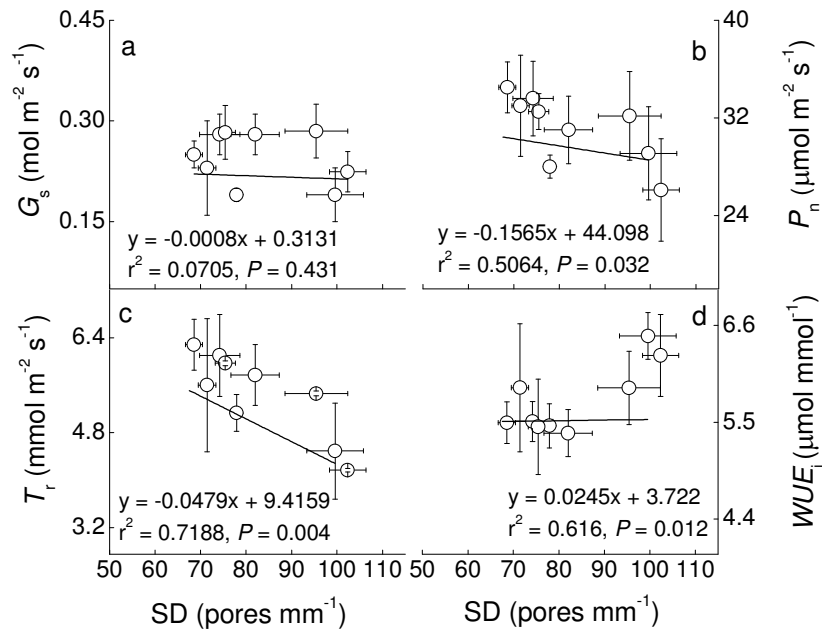
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524 **Fig. 7**

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