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# Shade and drought growth conditions strongly impact dynamic responses of stomata to variations in irradiance in *Nicotiana tabacum*

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24**Keywords: drought, dynamic response, irradiance, shade, stomatal conductance**

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26

## 27 Abstract

28 Recent research has made progress in describing stomatal dynamics in terms of speed, amplitude of  
29 response, lag time and response time. However, little is known about the impact of growth  
30 conditions on the rapidity of stomatal movements, and their relationship with stomatal morphology  
31 within a species. We measured stomatal dynamics during opening and closing in response to  
32 changes in irradiance in tobacco plants (*Nicotiana tabacum*) grown under “Control”, “Drought” and  
33 “Shade” treatments. Growth conditions strongly changed the rapidity of stomatal responses to  
34 irradiance. The “Drought” treatment considerably accelerated the response and “Shade” treatment  
35 slowed it down when compared to “Control”. We confirmed for the “Control” treatment the known  
36 asymmetry of response, with closing faster than opening, but interestingly the asymmetry  
37 disappeared under both treatments. Only stomatal density and index were affected by the growth  
38 conditions, not stomatal size and form. Thus, the observed variation in stomatal closing and opening  
39 dynamic parameters (speed, amplitude, lag time, response time) was not due to a variation in the  
40 size of the stomata, and only a marginal relationship between speed of the stomatal response and  
41 stomatal density was observed. These results suggest that physiological factors might be the main  
42 driver of variations in stomatal conductance dynamics within a species grown under different  
43 environmental conditions.

44

## 45 Highlights

- 46 • Growth conditions (drought/shade) change dynamics of stomatal response to irradiance
- 47 • Growth conditions change stomatal dynamics between lag and response times
- 48 • Growth conditions (drought/shade) change asymmetry between opening and closing
- 49 • Within species variation in stomatal speeds were not explained by stomatal size

## 501 Introduction

51 Stomatal morphology and their movements (opening and closing) are key components controlling  
52 exchange of water vapour and CO<sub>2</sub> between the leaf and the atmosphere, expressed as stomatal  
53 conductance ( $g_s$ ). Stomatal conductance per leaf surface is mainly determined by stomatal density,  
54 size, pore area (aperture) and their distribution. Since plants are subjected to a fluctuating  
55 environment through a diurnal cycle, with important variations of irradiance, vapour pressure deficit  
56 between the leaf and the atmosphere (VPD), temperature and soil water deficit, they need to balance  
57 gas exchange by adjusting stomatal conductance continuously during the day (Schulze and Hall,  
58 1992; Percy et al., 2000). Usually stomata respond to low CO<sub>2</sub>, low VPD and high irradiance by  
59 increasing their level of aperture, inducing an increase of  $g_s$  and vice versa (Outlaw, 2003).  
60 Nevertheless, these environmental changes usually occur concomitantly, making  $g_s$  a complex  
61 resultant of various signals which are induced via different signalling pathways and treated  
62 hierarchically (Lawson and Morison 2004; Lawson et al., 2010; Aasamaa and Sober, 2011;  
63 Haworth et al., 2018).

64 To describe the stomatal behaviour, numerous steady-state models of  $g_s$  have been proposed  
65 (reviewed in Damour et al., 2010). However  $g_s$  variations induced by environmental changes are not  
66 instantaneous and show a temporal response that can be described by dynamic models (Violet-  
67 Chabrand et al., 2017). Changes in stomatal aperture result from variations of water content of the  
68 guard cells, which are in turn produced by fluxes of potassium ions (K<sup>+</sup>) in or out of the guard cell  
69 (Blatt, 2000; Shimazaki et al. 2007). Kirschbaum et al. (1988) proposed a temporal model where the  
70 dynamic response of  $g_s$  to irradiance was first initiated by a biochemical signal responding to the  
71 environmental change, followed by an osmotic adjustment inside the guard cells resulting in  
72 stomatal opening. The combination of these processes described the response of  $g_s$  as a sigmoidal  
73 curve. The dynamic change of  $g_s$  to atmospheric environmental variations takes from a few minutes  
74 to almost an hour, depending on species and irradiance variation (Vico et al., 2011; McAusland et  
75 al., 2016). Compared to stomatal dynamics in response to changes in irradiance, the variation of net  
76 CO<sub>2</sub> assimilation ( $A_n$ ), if not limited by  $g_s$ , varies much faster, usually within a few seconds. Several  
77 studies highlighted the importance of photosynthetic response times for carbon uptake (reviewed in  
78 Kaiser et al., 2018), however as these are generally an order of magnitude faster than changes in  $g_s$ ,  
79 variation in the latter might be dominant in non-synchronicity situations.

80 Irradiance is the main environmental driver of photosynthesis, therefore the stomatal response to  
81 fluctuating light has been extensively studied (Kirschbaum et al., 1988 ; Shimazaki et al., 2007 ;  
82 Lawson et al., 2010 ; McAusland et al., 2016 ; Kardiman and Raebild, 2017 ; Matthews et al.,  
83 2018). Over time these fluctuations drive the temporal dynamics of carbon gain, water loss and by

84extension the water use efficiency of the plant (Lawson and Blatt, 2014). Stable environmental  
85conditions rarely occur in nature, therefore field measurements of  $g_s$  are unlikely to reach steady-  
86states values (Lawson et al., 2010) resulting in decoupled  $A_n$  and  $g_s$  measures and a non-  
87representative estimation of the ratio of  $A_n$  to  $g_s$ , the intrinsic water use efficiency (Lawson et al.,  
882010; McAusland et al., 2016, Vialet-Chabrand et al. 2017). A non-synchronicity in the temporal  
89response between  $A_n$  and  $g_s$  can have repercussions on carbon fixation, the water lost by  
90transpiration and long-term water use efficiency (McAusland et al., 2016). Due to the importance of  
91dynamic stomatal regulation, modelling of  $g_s$  responses to environmental changes can improve the  
92up-scaling of  $CO_2$  and water vapour exchange from the leaf to the canopy level (Vialet-Chabrand et  
93al., 2017). To parametrize such models, it is important to gain knowledge on the impact of growth  
94conditions on the stomatal dynamics.

95Morphological traits such as stomatal density and size regulate steady-state values of  $g_s$  (Franks and  
96Farquhar, 2001) and set the theoretically achievable maximum stomatal conductance by the plant  
97(Dow et al., 2014). A variation in stomatal morphology can lead to improved instantaneous and  
98long-term water use efficiency by impacting directly only  $g_s$ , but not  $A$  (Doheny et al., 2012; Franks  
99et al., 2015). It has also been shown that an increase in  $g_s$  under high irradiance conditions was  
100associated to an increase of stomatal density (Schlüter et al., 2003). Stomatal morphology and  
101patterning is known to be influenced by both environmental growth conditions and plant hormones  
102(Woodward, 1987; Hetherington and Woodward, 2003; Casson and Gray, 2008; Kardiman and  
103Raebild, 2017). In tobacco leaves, Thomas et al. (2004) observed a 12.7-24.2% decrease of stomatal  
104index (ratio between number of stomata to total number of stomatal and epidermal cells) of  
105developing leaves exposed to shading compared to a control treatment. Although growth conditions  
106varying in atmospheric  $CO_2$  concentration and irradiance have been shown to impact stomatal  
107morphology, little is known about other environmental factors such as soil water stress. Jones  
108(1977) showed for barley that a reduction of soil water availability could result in a decrease in  
109stomatal index, but this is not always consistent as reported for groundnut (Clifford et al., 1995),  
110where the stomatal index was not changed by water stress.

111It has been hypothesized (Hetherington and Woodward, 2003; Drake et al. 2013, Raven, 2014;  
112Kardiman and Raebild 2017) that stomatal traits such as density and size might be involved in the  
113temporal response of  $g_s$  to an environmental change. These studies, based mainly on among species  
114comparisons, suggested that a smaller stomatal size resulted in a faster stomatal response due to the  
115higher surface-to-volume ratio and the lower subsequent solute transport required to drive stomatal  
116movements (Lawson et al., 2014). Other recent evidence using a very large spectrum of species,  
117including ferns, cycads, conifers, and angiosperms (Elliot-Kingston et al., 2016) had not found a

118relationship between stomatal size and closing speed, however the authors suggest a relationship  
119with the atmospheric CO<sub>2</sub> levels during species diversification. Moreover, variations in stomatal  
120size are often negatively correlated to variations in stomatal density (Franks and Beerling, 2009;  
121Doheny-Adam et al., 2012). Also, the relationship between stomatal response times and the shape  
122of the guard cells (Hetherington and Woodward, 2003; Franks and Farquhar, 2007; McAusland et  
123al., 2016) and their patterning (Papanatsiou et al., 2016) have been studied. It has been shown that  
124the stomatal shape (dumbbell or elliptical) might be a determinant driver of stomatal speed as  
125dumbbell-shaped guard cells species tend to display faster responses to environmental fluctuations  
126(Hetherington and Woodward, 2003; MacAusland et al., 2016). Moreover, the stomatal shape and  
127patterning might confer different mechanical advantages via cell osmotic and turgor pressures  
128influencing the rapidity of stomatal response (Franks and Farquhar, 2007). However, other  
129parameters than stomatal morphology, such as variations in ion and water transport within guard  
130cells, are likely to impact the speed of stomatal responses (Lawson and Blatt, 2014).

131Growth conditions such as shade and soil water deficit have been shown to determine steady state  
132stomatal conductance, however, only a few studies examined their influence on stomatal dynamics.  
133In these studies, drier conditions have been related to faster stomatal response to irradiance (Vico et  
134al., 2011; Lawson and Blatt., 2014; Qu et al., 2016) suggesting an influence of plant water balance  
135on the rapidity of the stomatal response. Martins et al. (2016) demonstrated in conifers and ferns a  
136major impact of leaf hydraulic status on g<sub>s</sub> response time to variations in vapour pressure deficit.  
137Less is known about the impact of different light environments during growth on the dynamics of  
138stomatal responses to a change in irradiance. Matthew et al. (2018) have shown faster stomatal  
139responses with an increased amplitude for *Arabidopsis thaliana* grown under fluctuating high light,  
140compared to plants grown under shaded conditions. Kardiman and Raebild (2017) showed that,  
141although the dynamics of stomata of most tested species remained unaffected by their light  
142environment, early successional species displayed faster stomatal responses when grown under  
143shade conditions compared to the ones grown under full light, while late successional species  
144displayed the opposite behaviour. These results could suggest that the acclimation of stomatal  
145dynamics to the light environment might be species specific and related to their ecology.

146Differences in the speed of stomatal dynamics to a variation in irradiance have been shown among  
147species and within individuals of the same species (Vico et al., 2011; McAusland et al., 2016).  
148Stomatal speed has also been linked to plant functional types and environmental factors (Vico et al.,  
1492011): graminoids tended to display shorter responses than forbs, woody gymnosperms or  
150angiosperms and plants from dryer climates seemed to exhibit faster responses. Moreover, many  
151species show an asymmetric response between stomatal opening and closing, ie, over 60% of the

152species reviewed by Ooba and Takahashi (2003) displayed a faster opening. Ooba and Takahashi  
153(2003) argued that such an asymmetry could be related to the environmental growth conditions of  
154the different species, where a light limited environment might favour a more rapid opening of  
155stomata. However, Woods and Turner (1971) have suggested that a faster stomatal closure would  
156reduce the temporal decoupling between  $A_n$  and  $g_s$  while a slower opening would reduce water loss  
157without reducing  $A_n$  when  $g_s$  is not limiting, especially under well-watered conditions. Such a  
158temporal asymmetry of stomatal movements might lead to a reduced transpiration and thus translate  
159a conservative stomatal behaviour. Moreover, a slow opening might prevent situations of a  
160continued  $g_s$  increase after  $A_n$  has reached light saturation, which would result in an excessive water  
161loss compared to carbon gain (Kirschbaum et al., 1988; Lawson et al., 2010; Vialet-Chabrand et al.,  
1622017). The literature therefore suggests that the asymmetry between opening and closing of stomata  
163might have an important ecological impact, depending on the growth conditions.

164

165Thus, our main objectives were to analyse:

- 166 (i) the impact of three different growth conditions (control, shade and drought) on the  
167 parameters conditioning the temporal response of  $g_s$  to step variations in irradiance;
- 168 (ii) the relationship between stomatal morphology and the dynamics of stomatal responses

169The dynamics of the temporal stomatal response to a step variation in irradiance was characterised  
170by a model based on Vialet-Chabrand et al. (2013), which decomposes the sigmoidal response into  
171a) the delay of stomatal response, b) the response time constant and c) the amplitude of stomatal  
172movements. From the latter two parameters a maximal speed can be calculated. The stomatal  
173response has been tested for opening and closing, which allowed also to estimate the asymmetry for  
174the dynamic parameters.



## 1752 Material and methods

### 1762.1 Plant material and Experimental design

177The experiment lasted for 8 weeks and was carried out on eighteen plants of *Nicotiana tabaccum* L.  
178wild type (cv. Petite Havana SR1), grown at the University of the Balearic Island (UIB), Palma,  
179Spain, 39°38'11.9"N 2°38'49.7"E in autumn 2015. Seeds were germinated in petri boxes with  
180humidified filter paper and after one week transferred into 2L pots filled with 1/3 (V/V) of perlite  
181and organic soil, respectively.

182The two weeks old seedlings were then randomly divided into the three treatments: "Control" (6  
183plants), "Shade" (7 plants) and "Drought" (5 plants). The "Control" treatment was characterized by  
184ambient growth irradiance ( $400\text{-}450 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) and well-watered conditions. The  
185"Shade" treatment was characterized by low irradiance ( $40 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ) and the same well-  
186watered conditions as in the "Control" treatment. The irradiance compensation point, estimated  
187from steady-state irradiance response curves (data not shown) were estimated at  $30 \mu\text{mol m}^{-2} \text{ s}^{-1}$  for  
188"Control" and  $27 \mu\text{mol m}^{-2} \text{ s}^{-1}$  for "Shade" treatments; 90% of maximum A was reached at about  
189 $1100 \mu\text{mol m}^{-2} \text{ s}^{-1}$  for "Control" and at about  $990 \mu\text{mol m}^{-2} \text{ s}^{-1}$  for "Shade"; 90% of maximum  $g_s$  for  
190"Shade" was reached at  $500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ . Plants in "Control" and "Shade" treatments were watered  
191every second day with Hoagland's solution, 50% dilution. The "Drought" treatment was  
192characterized by the same ambient growth irradiance as the "Control" treatment, but the plants were  
193submitted to a soil water deficit. The water deficit was controlled by weighing the pots at field  
194capacity and watering to 50% of the weight. During the experiment the field capacity weight was  
195verified on the plants of the "Control" treatment on a regular basis and the difference assumed to be  
196the plant growth and added to the target weights of the "Drought" treatment. Before application of  
197the treatments, the last emergent leaf was marked to ensure gas exchange measurement were done  
198on leaves grown under treatment conditions. Other conditions in the growing chamber were 25°C,  
199air relative humidity 50-60% and a photoperiod of 12h/12h (8:00-20:00).

### 2002.2 Measurement of leaf water potential

201To determine the plant water status, midday leaf water potential ( $\Psi$ ) was measured in fully  
202expanded leaves with a Scholander pressure chamber (Soil moisture Equipment Corp., Santa  
203Barbara, CA, USA). At the end of the experiment, that is six weeks after separating the plants into  
204the treatments, six leaves were measured for the control treatment from different plants and 4 leaves  
205for each, the shade and the drought treatment.

### 2062.3 Gas exchanges measurements

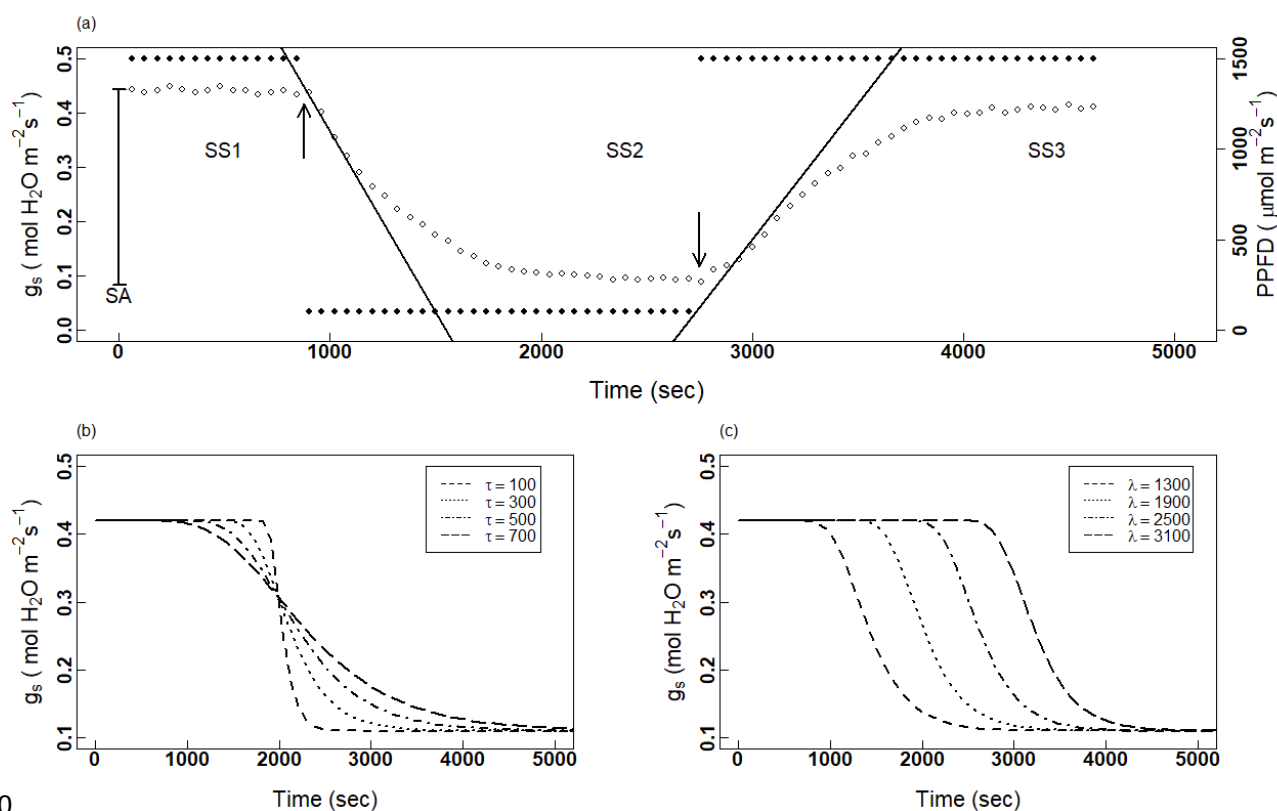
Comment citer ce document :

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207 Gas exchange was measured using a portable photosynthesis system (LI-COR 6400; LI-COR,  
208 Lincoln, NE, USA) equipped with a 2cm<sup>2</sup> leaf chamber (Li-6400-40). Measured were: net CO<sub>2</sub>  
209 assimilation rate ( $A_n$ ), stomatal conductance for water vapour ( $g_s$ ) and leaf internal CO<sub>2</sub>  
210 concentration ( $C_i$ ) (see Table 1 for units). All measurements were carried out between 10:00 and  
211 19:00 h (Central European summer time). For each plant measured, gas exchange measurements  
212 were performed on the youngest, mature, fully expanded leaf, which had grown under treatment  
213 conditions. This leaf has been measured three times at different days and different times during the  
214 day. Overall, the measurement of all stomatal response curves has taken 18 days. The  
215 environmental parameters inside the chamber were kept constant during the acclimation phase with  
216 [CO<sub>2</sub>] entering the chamber of 400  $\mu\text{mol mol}^{-1}$ , block temperature of 25°C, air flow of 300  $\mu\text{mol}$   
217  $\text{min}^{-1}$  and a PPFD of 1500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (red/blue irradiance 90/10%, respectively) until the leaf  
218 reached a steady-state of  $g_s$  (SS1; Fig. 1a). Then a measurement cycle consisted of two step-changes  
219 in irradiance: first I) a single step-change to low irradiance inducing stomatal closure and then II) a  
220 single step-change back to the original high irradiance, inducing a stomatal reopening. For the low  
221 irradiance step, the PPFD was lowered from 1500 to 100  $\mu\text{mol.m}^{-2} \text{s}^{-1}$  until the plant reached a new  
222 steady-state (SS2). After 10 minutes under this new steady-state, the PPFD was set back to its initial  
223 setting at 1500  $\mu\text{mol.m}^{-2} \text{s}^{-1}$  and measurements were recorded until a new steady state was reached  
224 (SS3). The stomata were considered in steady-state when  $g_s$  did not vary more than  $\sim 0.005 \text{ mol m}^{-2}$   
225  $\text{s}^{-1}$  during 10min. This resulted in a standard deviation over the 10 minutes of 0.0015  $\text{mol m}^{-2} \text{s}^{-1}$ .  
226 Data during the response curves were logged every 60sec. “Steady-state” data as mentioned through  
227 the manuscript were calculated for SS1, SS2 and SS3 as the mean of 5 points after stabilization of  
228  $g_s$ , (Fig. 1a).

229



230

231 Figure 1: Illustration of the stomatal dynamics for irradiance step-changes. a: Example of a  
 232 measured stomatal closure and opening (white dots) provoked by a change in irradiance (black  
 233 dots). Black arrows represent the irradiance changes, the black lines are the maximal slopes ( $SL_{\max}$ )  
 234 of both opening and closing sequences and the amplitude of the stomatal response (SA). b:  
 235 Simulation of the impact of increasing values by 200secs steps of  $\tau$  (response time) on the  
 236 curvature of the sigmoidal model, and c: Simulation of the impact of 600secs steps increasing  
 237 values of  $\lambda$  (lag time) on the stomatal delay.

238

#### 239 2.4 Stomata morphology

240 At the end of the experiment one leaf was sampled from each of five plants per treatment to  
 241 determine the stomatal morphology of leaves on the abaxial and adaxial faces. Specifically, the  
 242 following parameters were measured : the stomatal density (SD), epidermal cell density (CD),  
 243 stomatal index (SI) defined as  $SD/(SD+CD)$ , length of the stomatal guard cell complex (GCL),  
 244 guard cells width (GCW), stomatal surface (SS) defined as an ellipse area:  $\pi \cdot (GCL/2) \cdot (GCW/2)$   
 245 and guard cell shape (GSH) defined as the ratio  $GCL/GCW$ .  $1\text{cm}^2$  portions of the leaves were  
 246 collected and nail polish imprints of both leaf surfaces were taken using adhesive film and applied  
 247 on microscope slides for analysis. Stomata and epidermis cells were counted in the obtained images

248 using the ImageJ2 software (Schindelin et al. 2015). Six images (500\*370µm) of the abaxial and  
249 adaxial surfaces of the leaf used for gas exchange were taken.

### 250 2.5 Model description

251 The stomatal responses of the irradiance curves were adjusted using a sigmoidal model based on  
252 Vialet-Chabrand et al. (2013). The sigmoidal model allows the estimation of parameters describing  
253 the temporal response of the stomata to an environmental change. The following equation was used:

$$254 g_s = r_0 + (G - r_0) * \exp\left(-\exp\left(\frac{\lambda - t}{\tau}\right)\right)$$

255 where  $g_s$  is the fitted stomatal conductance,  $r_0$  is the starting value of the stomatal conductance (first  
256 steady-state obtained after the plant acclimation to the environmental conditions inside the Licor  
257 chamber,  $g_{min}$  or  $g_{max}$ ),  $G$  the ending value of stomatal conductance (second steady-state reached  
258 after the full stomatal response to the irradiance change,  $g_{max}$  or  $g_{min}$ ),  $\lambda$  is the lag time of the  
259 stomatal response (time needed to reach the inflection point of the curve from the moment of the  
260 irradiance change in each curve), and  $\tau$  the response time. Compared to the sigmoidal equation used  
261 by Vialet-Chabrand et al. (2013), here,  $\lambda$  is mathematically independent from  $\tau$  (see Fig. 1 b and c).  
262 From these parameters, the maximum slope ( $SL_{max}$ ) as estimator of the speed of the stomatal  
263 response, can be calculated as:

$$264 SL_{max} = (1/\tau) * (G - r_0) / e$$

265 Where  $(G-r_0)$  represents the amplitude of the stomatal response (SA) and  $e$  the Euler constant.  
266 Increasing values of  $\tau$  will affect the curvature of the stomatal response, the smaller a  $\tau$  value is, the  
267 stronger the curvature (Fig. 1b) and the higher  $SL_{max}$  will be, so the more rapidly  $g_s$  will  
268 increase/decrease.

269 This model was adjusted using the function “nlminb” of R (Team RC, 2015). To facilitate the  
270 adjustment of the sigmoidal model, five data points during the steady state before changing the  
271 irradiance were first included in the model adjustment. This affects only the lag time  $\lambda$ , which was  
272 then corrected by subtracting the added time period. The model adjustment is sensitive to the  
273 starting point and including five steady state points made the starting steady state  $g_s$  more robust and  
274 decreases the dependency of the adjustment on measurement noise.

275 As SS1 and SS3 values for all gas exchange variables were not significantly different (pairwise t-  
276 test  $p > 0.05$ ) the mean amplitude between closing and opening in response to the step-change in  
277 irradiance were calculated for  $A_n$  and  $g_s$  as absolute and relative values (SA and RSA, respectively;  
278 Table 1) :

$$279SA_{An} = ((An\ SS1 - An\ SS2) + (An\ SS3 - An\ SS2)) / 2$$

$$280SA_{gs} = ((g_s\ SS1 - g_s\ SS2) + (g_s\ SS3 - g_s\ SS2)) / 2$$

$$281RSA_{An} = (((An\ SS1 - An\ SS2) / An\ SS1) + ((An\ SS3 - An\ SS2) / An\ SS3)) / 2 * 100\%$$

$$282RSA_{gs} = (((g_s\ SS1 - g_s\ SS2) / g_s\ SS1) + ((g_s\ SS3 - g_s\ SS2) / g_s\ SS3)) / 2 * 100\%$$

283For the adjusted dynamic parameters, we also calculated the ratio between closing and opening,  
284representing the asymmetry of the response. Further, the ratio between  $\tau$  and  $\lambda$  was calculated, to  
285characterise the relative impact of different treatments on both parameters.

286

### 2872.6 Statistical analysis

288All statistical analyses were performed with R (Team RC, 2015). Treatment effects were analysed  
289as a one factorial design analysis of variance (ANOVA). Significant differences were considered at  
290 $p < 0.05$ . When the ANOVA showed a significant treatment effect, a Post-Hoc test using Tukey-  
291HSD (package R, “agricolae”) was used to estimate significance of inter-group differences.  
292Comparisons among steady-state gas exchange variables as well as of closing versus opening were  
293done using pairwise t-tests. For all stomatal morphology traits, mean values of all images taken on  
294the adaxial and abaxial surfaces were used for ANOVA and correlations with gas exchange results  
295were based on a mean of the 3 repetitions per leaf. Correlations were estimated using the Pearson  
296method and p-values were adjusted for multiple comparisons using the “p.adjust” function with the  
297“FDR” method.

## 2983 Results

### 2993.1 Steady-state gas exchange parameters under three different growth conditions

300The different growth conditions significantly affected  $\Psi$  of tobacco plants, with the highest value  
301for the “Shade” treatment ( $-0.37 \pm 0.06$  Mpa), without being significantly different from the  
302“Control” treatment ( $-0.51 \pm 0.09$  Mpa,  $p > 0.05$  TukeyHSD), while the “Drought” treatment  
303significantly decreased  $\Psi$  to  $-1.46 \pm 0.18$  Mpa ( $p > 0.05$  TukeyHSD).  $A_n$  values measured under  
304saturating irradiance (SS1, see Fig. 1) were significantly different among treatments, where the  
305“Control” treatment showed the highest value and “Shade” the lowest (Table 1).  $g_s$  values differed  
306among the treatments, showing the highest values in “Control” and the lowest in “Drought”. These  
307strong differences for  $A_n$  and  $g_s$  were associated with significantly different  $C_i$  among treatments,  
308with the highest  $C_i$  in the “Shade” and the lowest under “Drought”. Decreasing irradiance from high  
309to low intensity (SS1 to SS2, see Fig. 1),  $A_n$  and  $g_s$  decreased drastically as expected (Table 1), but  
310not  $C_i$ , for which no significant differences were detected among the three steady-states, within each  
311treatment.

### 3123.2 Amplitude of response under changing measuring irradiance

313For both  $A_n$  and  $g_s$ , their respective variations were similar during closing and opening as there was  
314no significant difference between the two high-irradiance steady state measurements (SS1 and SS3;  
315pairwise t-test  $p > 0.05$ ) indicating a full reopening, therefore the analysis of the absolute and  
316relative amplitude of the responses (SA and RSA (%), respectively) was based on the mean values  
317of closing and opening sequences (presented in Table 1). The absolute amplitude of the A response  
318( $SA_{A_n}$ ) was highest for “Control” and lowest for “Shade”, where the absolute amplitudes for  $g_s$   
319( $SA_{g_s}$ ) were highest for “Control”, but lowest for “Drought”. Although the SA were significantly  
320different among treatments for both A and  $g_s$ , the relative amplitudes (RSA) were only significantly  
321lower for the “Shade” treatment for both traits.

### 3223.3 Dynamic parameters of stomatal response under irradiance changes

323During the closing sequence (from SS1 to SS2), the response time ( $\tau$ ) ranged from  $\sim 90$  s for the  
324faster responses to  $\sim 600$  s for the slowest (Table 2). Each treatment was significantly different from  
325each other, with “Shade” (slowest) < “Control” < “Drought” (fastest). Interestingly, we only  
326observed significantly different  $\tau$  values between opening and closing sequence for the “Control”  
327treatment, with an asymmetrical response of  $\sim 2$  times slower opening, whereas “Drought” and  
328“Shade” did not show a significantly asymmetric responses.

329

330Table 1: Means per treatment ( $\pm$  standard error of the mean) of net CO<sub>2</sub> assimilation ( $A_n$ ), stomatal  
 331conductance ( $g_s$ ), internal CO<sub>2</sub> concentration ( $C_i$ ) at each steady-state reached during the  
 332measurement cycle (SS1-SS2-SS3, see Figure 1a for details). The amplitude of variation of the  
 333parameters between each steady state (SA and RSA, respectively in absolute values and  
 334percentage). For  $C_i$ , amplitude of variations was not shown as no differences between the steady  
 335states were observed. Different letters show the significant differences among treatments using a  
 336Tukey-HSD test. “\*” represents the significant differences between two steady states from a paired  
 337t-test at  $p < 0.05$ . There were no significant differences between SS1 and SS3, which were therefore  
 338not indicated in the table.

Treatments		SS1		SS2		SS3		SA		RSA (%)
Control	$A_n$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	$19.9 \pm 0.4^a$ (15)	*	$4.7 \pm 0.4^a$ (16)	*	$20.3 \pm 0.4^a$ (16)		$15.5 \pm 0.2^a$ (15)		$77.0 \pm 1.6^a$ (15)
Drought		$11.1 \pm 0.5^b$ (18)	*	$2.8 \pm 0.2^b$ (17)	*	$12.1 \pm 0.7^b$ (17)		$8.9 \pm 0.7^b$ (17)		$75.2 \pm 2.9^a$ (17)
Shade		$8.5 \pm 0.5^c$ (18)	*	$3.7 \pm 0.2^{ab}$ (18)	*	$7.9 \pm 0.7^c$ (18)		$4.6 \pm 0.6^c$ (18)		$54.2 \pm 2.8^b$ (18)
Control	$g_s$ ( $\text{mol m}^{-2} \text{s}^{-1}$ )	$0.41 \pm 0.03^a$ (15)	*	$0.11 \pm 0.01^a$ (16)	*	$0.38 \pm 0.03^a$ (16)		$0.28 \pm 0.02^a$ (15)		$72.6 \pm 1.6^a$ (15)
Drought		$0.08 \pm 0.00^c$ (18)	*	$0.02 \pm 0.01^b$ (17)	*	$0.09 \pm 0.01^c$ (17)		$0.06 \pm 0.01^c$ (17)		$71.9 \pm 0.9^a$ (17)
Shade		$0.22 \pm 0.01^b$ (18)	*	$0.1 \pm 0.01^a$ (18)	*	$0.2 \pm 0.01^b$ (18)		$0.11 \pm 0.01^b$ (18)		$54.2 \pm 2.4^b$ (18)
Control	$C_i$ ( $\mu\text{mol mol}^{-1}$ )	$292.0 \pm 5.5^b$ (15)	ns	$312.5 \pm 9^a$ (16)	ns	$286.4 \pm 5.9^a$ (16)				
Drought		$160.3 \pm 11.9^c$ (18)	ns	$193.5 \pm 11^b$ (17)	ns	$159.7 \pm 15.9^b$ (17)				
Shade		$320.3 \pm 4.4^a$ (18)	ns	$323.5 \pm 4.1^a$ (18)	ns	$316.0 \pm 7.7^a$ (18)				

339

340Table 2: Dynamic parameters for the opening and closing sequences, with  $\tau$  the response time,  $\lambda$  the  
 341delay of stomatal response,  $\tau/\lambda$  their ratio and  $SL_{\text{max}}$  the maximal slope of the response. Different  
 342letters show the significant differences between treatments from an ANOVA model including  
 343treatment effects followed by a post-hoc Tukey test. The number of analysed response curves are in  
 344parentheses, with a mean of 2.7 repetitions per leaf. \* show the significant differences between  
 345closing and opening from a paired t-test (P-values  $< 0.05$ ), so a significant difference indicates an  
 346asymmetric response.

Treatment		Closing SS1-SS2		Opening SS2-SS3		Ratio Closing/Opening
Control	$\tau$ (sec)	$378 \pm 38^a$ (15)	*	$695 \pm 118^a$ (16)		$0.59 \pm 0.04^b$ (15)
Drought		$87 \pm 4^c$ (18)	ns	$101 \pm 18^b$ (17)		$1.09 \pm 0.16^a$ (17)
Shade		$577 \pm 38^b$ (18)	ns	$637 \pm 52^a$ (18)		$0.92 \pm 0.06^a$ (18)
Control	$\lambda$ (sec)	$348 \pm 15^a$ (15)	*	$693 \pm 41^a$ (16)		$0.51 \pm 0.01^a$ (15)
Drought		$151 \pm 9^c$ (18)	*	$430 \pm 81^b$ (17)		$0.39 \pm 0.03^b$ (17)
Shade		$278 \pm 16^b$ (18)	*	$495 \pm 21^b$ (18)		$0.57 \pm 0.02^a$ (18)
Control	$\tau/\lambda$	$1.08 \pm 0.08^b$ (15)	ns	$0.99 \pm 0.13^a$ (16)		
Drought		$0.58 \pm 0.04^c$ (18)	*	$0.25 \pm 0.05^b$ (17)		
Shade		$2.09 \pm 0.1^a$ (18)	*	$1.3 \pm 0.12^a$ (18)		
Control	$SL_{\text{max}}$ ( $\text{mol m}^{-2} \text{s}^{-2} \times 10^5$ )	$-3.13 \pm 0.4^b$ (15)	*	$1.79 \pm 0.16^b$ (16)		$-1.84 \pm 0.11^b$ (15)
Drought		$-3.16 \pm 0.3^b$ (18)	*	$3.72 \pm 0.59^a$ (17)		$-0.97 \pm 0.11^a$ (17)
Shade		$-0.96 \pm 0.1^a$ (18)	*	$0.75 \pm 0.06^b$ (18)		$-1.33 \pm 0.06^a$ (18)

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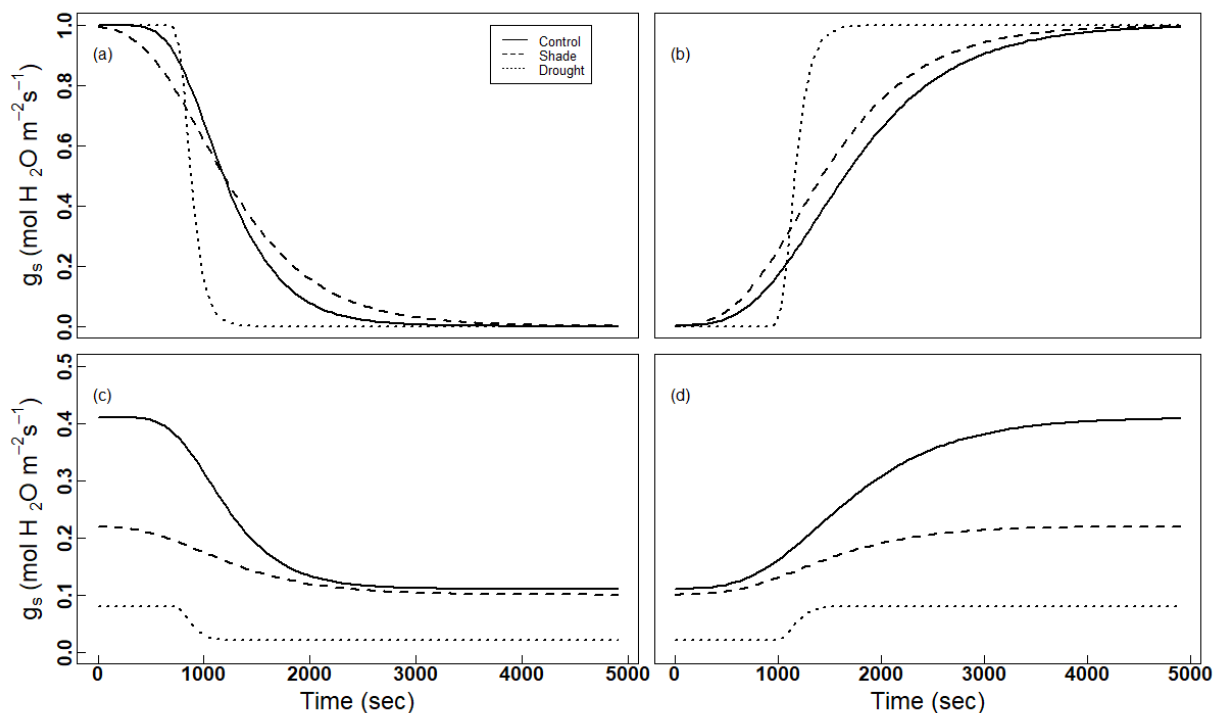
348The lag time of the response ( $\lambda$ ) showed a different pattern compared to  $\tau$ . During the closing  
349sequence, “Drought” showed again the shortest delay (the fastest response, lowest  $\lambda$  values), then  
350“Shade” treatment, then “Control” (the longest delay, highest  $\lambda$  values). In all treatments,  $\lambda$  was  
351significantly higher for the opening than for the closing sequence (Table 2), with the strongest  $\lambda$   
352asymmetry for the “Drought” treatment while the ratios were similar between “Shade” and  
353“Control” treatments.

354For the “Control” treatments,  $\lambda$  and  $\tau$  showed very similar values (ratio  $\tau/\lambda \sim 1$ ) in both closing and  
355opening sequences, for “Drought” and “Shade” treatments a significant deviation from unity was  
356observed but in opposite directions: the “Drought” treatment induced a shift to a longer  $\lambda$ , whereas  
357the “Shade” treatment induced a shift to higher  $\tau$ .

358  $SL_{\max}$  for closing was significantly slower in the “Shade” treatment, whereas for opening it was  
359significantly faster for the “Drought” treatment. This was due to the strong asymmetry observed for  
360the “Control” treatment, which was much smaller in the “Drought” and “Shade” treatments.

361In Figure 2, the mean of the estimated parameters for each treatment was applied to the sigmoidal  
362model to visualize the differences in the responses, using a normalized  $g_s$  scale (setting  $g_{\min}$  to 0 and  
363 $g_{\max}$  to 1; Fig. 2a and Fig. 2b), as well as the measured  $g_s$  values (Fig. 2c and 2d). Plants from the  
364“Drought” treatment reached the new steady state after the step-change in irradiance significantly  
365more rapidly compared to the other two treatments. As the normalized graphs do not depend on the  
366amplitude, they illustrate the difference between the “Control” and “Drought” treatments in terms of  
367 $\tau$  and  $\lambda$  : for a similar overall response time, the “Shade” treatment showed a shorter time lag of the  
368response.





370  
 371 Figure 2: Fitted stomatal dynamics induced by changes in light intensity in closing (a, c) and  
 372 opening (b, d) sequences. Plain lines are for “Control”, simple dashed lines for “Shade” and dotted  
 373 lines for “Drought” treatment. Figures (a) and (b) show normalized conductance responses ( $g_{\min} = 0$ ,  
 374  $g_{\max} = 1$ ), whereas measured values of  $g_{\min}$  and  $g_{\max}$  were used for Figures (c) and (d). Each curve  
 375 was estimated by using the mean values of the dynamic parameters from Table 2 and the sigmoidal  
 376 model.

377

#### 378 3.4 Stomatal morphology in response of different treatments

379 No significant differences were found between abaxial and adaxial faces for the considered stomatal  
 380 traits. Overall, the stomatal ratio abaxial/adaxial for the measured tobacco plants was  $2.46 \pm 0.4$   
 381 while the epidermal cell ratio was lower at  $1.39 \pm 0.1$ .

382 Significantly lower SD and CD as well as SI values were observed for the “Shade” treatment (Table  
 383), whereas no significant differences among treatments were observed for GCW, GSH and SS,

384

385 Table 3: Stomatal morphology means for treatments ( $\pm$  standard error of the mean). GCL: guard cell  
 386 length ( $\mu\text{m}$ ), GCW: pore width ( $\mu\text{m}$ ), SS: stomatal surface ( $\mu\text{m}^2$ ), GSH: stomatal shape  
 387 (GCL/GCW), SD: stomatal density ( $\text{mm}^{-2}$ ), CD: epidermis cell density ( $\text{mm}^{-2}$ ), SI: stomatal index  
 388 (SD/SD+CD). Different letters show the significant differences between the treatments (Tukey-  
 389 HSD).

Treatment	Trait	Mean $\pm$ SE (N)
Control	GCL ( $\mu\text{m}$ )	33.1 $\pm$ 1.2 <sup>a</sup> (5)
Drought		35.1 $\pm$ 1.7 <sup>a</sup> (5)
Shade		34.0 $\pm$ 0.5 <sup>a</sup> (5)
Control	GCW ( $\mu\text{m}$ )	26.7 $\pm$ 0.8 <sup>a</sup> (5)
Drought		26.4 $\pm$ 0.8 <sup>a</sup> (5)
Shade		24.1 $\pm$ 0.3 <sup>a</sup> (5)
Control	SS ( $\mu\text{m}^2$ )	695 $\pm$ 55.2 <sup>a</sup> (5)
Drought		731 $\pm$ 73 <sup>a</sup> (5)
Shade		666 $\pm$ 22 <sup>a</sup> (5)
Control	GSH	1.24 $\pm$ 0.02 <sup>b</sup> (5)
Drought		1.33 $\pm$ 0.03 <sup>ab</sup> (5)
Shade		1.41 $\pm$ 0.02 <sup>a</sup> (5)
Control	SD ( $\text{mm}^{-2}$ )	126 $\pm$ 7 <sup>a</sup> (5)
Drought		131 $\pm$ 12 <sup>a</sup> (5)
Shade		58 $\pm$ 8 <sup>b</sup> (5)
Control	CD ( $\text{mm}^{-2}$ )	478 $\pm$ 15 <sup>a</sup> (5)
Drought		544 $\pm$ 73 <sup>a</sup> (5)
Shade		318 $\pm$ 25 <sup>b</sup> (5)
Control	SI	0.2 $\pm$ 0.01 <sup>a</sup> (5)
Drought		0.19 $\pm$ 0.01 <sup>a</sup> (5)
Shade		0.15 $\pm$ 0.01 <sup>b</sup> (5)

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391 Table 4. Correlation table of dynamic parameters and the stomatal morphology across treatments. The upper-right triangle displays the p-values (with  
 392 “\*\*\*” for  $p < 0.001$ ; “\*\*” for  $p < 0.01$  and “\*” for  $p < 0.05$ , corrected for multiple comparisons using FDR), while the lower-left displays the r-values  
 393 (Pearson test). With the dynamic parameters:  $\tau$ ,  $\lambda$ ,  $SL_{max}$  (where closing slopes are negative), their closing/opening ratio, the absolute amplitude of  
 394 stomatal conductance response (SA), and the stomatal parameters: GCW (guard cells width), GCL (guard cells length), SS (stomatal size), SD  
 395 (stomatal density), and SI (the stomatal index).  $N=18$  for within dynamic parameter correlations and  $N=15$  for correlations with morphology traits, bold  
 396 r-values are highly significant (\*\*\*).

	$\tau_{cl}$	$\tau_{op}$	$\lambda_{cl}$	$\lambda_{op}$	$SI_{max\ cl}$	$SI_{max\ op}$	$\tau_{ratio}$	$\lambda_{ratio}$	$SL_{max\ ratio}$	$SA_{cl}$	$SA_{op}$	GCW	GCL	SS	SD	SI
$\tau_{cl}$		***	**		***	***		**							*	*
$\tau_{op}$	<b>0.83</b>		***	***	*	***	**	*	***	*						
$\lambda_{cl}$	0.66	<b>0.87</b>		***		*	**	*	***	**	*					
$\lambda_{op}$	0.46	<b>0.75</b>	<b>0.9</b>			*	**		***	**	**					
$SI_{max\ cl}$	<b>0.78</b>	0.48				***		*							*	**
$SI_{max\ op}$	<b>-0.85</b>	<b>-0.76</b>	-0.58	-0.47	<b>-0.78</b>		*	.	*							
$\tau_{ratio}$		-0.62	-0.67	-0.69		0.55			***		**					
$\lambda_{ratio}$	0.68	0.51	0.48		0.48	-0.44										*
$SL_{max\ ratio}$		<b>-0.71</b>	<b>-0.79</b>	<b>-0.84</b>		0.54	<b>0.83</b>			**	**					
$SA_{cl}$		0.59	0.84	0.85					-0.77		***					
$SA_{op}$			0.77	0.82			-0.69		0.69	<b>0.96</b>						
GCW													**	***		*
GCL												0.68		***		
SS												<b>0.9</b>	<b>0.93</b>			
SD	-0.63					-0.64										**
SI	-0.52					-0.69		-0.54				0.64		0.45	0.72	

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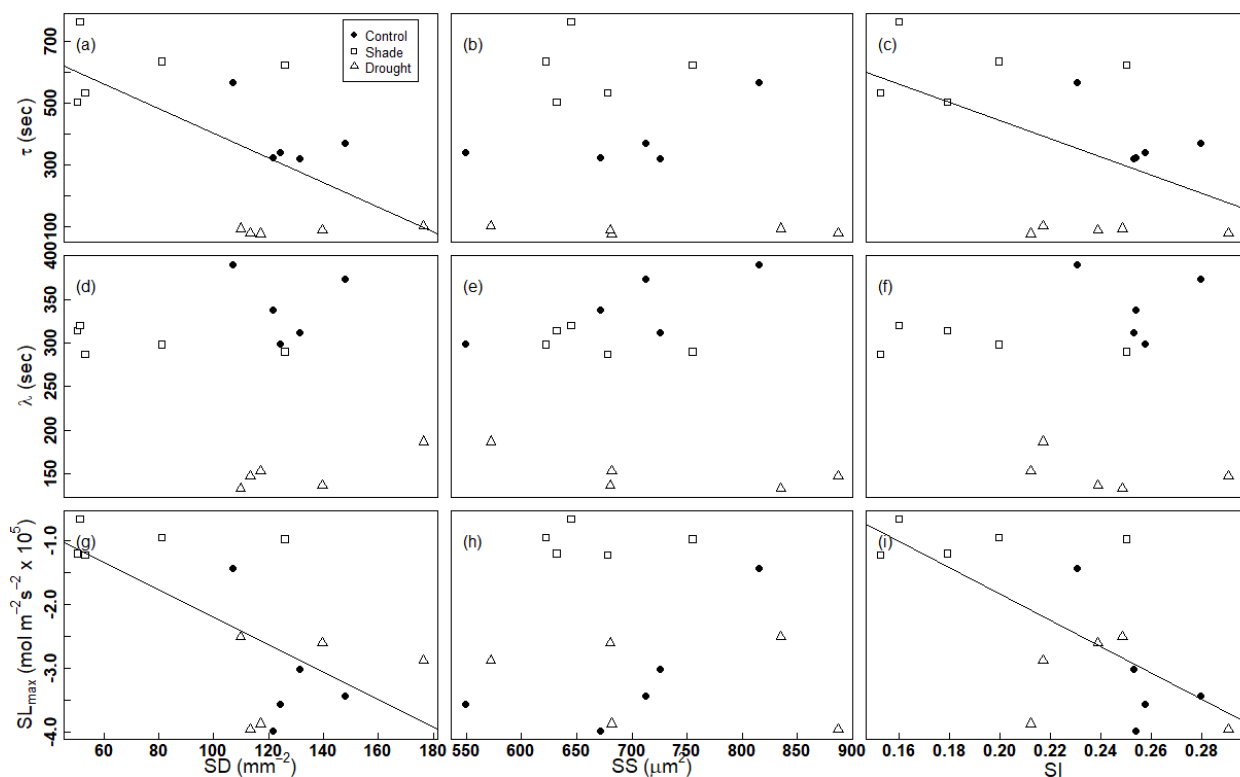
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### 3973.5 Cross-correlations between stomatal dynamics and morphology

398The dynamic parameters  $\tau$ ,  $\lambda$ ,  $SL_{max}$  and SA displayed significant and high correlations between  
 399their opening and closing values (Table 4).  $\tau$ ,  $\lambda$  and  $SL_{max}$  also correlated with each other, especially  
 400within the same irradiance change sequences, except for  $\lambda$  vs.  $SL_{max}$  where the correlations were  
 401lower or not significant during opening and closing respectively. However, within the same  
 402sequence, SA did only correlate with  $\lambda$  and not with  $\tau$  or  $SL_{max}$ .

403There were no correlations between  $\tau$ ,  $\lambda$ ,  $SL_{max}$ , SA and the stomatal size parameters (GCW, GCL,  
 404SS). For closure,  $\tau$  and  $SL_{max}$  showed small negative correlations with SD and SI (Fig. 3),  
 405associating more stomata with faster responses. The  $\tau$  relationships were more clearly driven by the  
 406treatments differences than the  $SL_{max}$  relationships (Fig. 3). Whereas  $\lambda$  did not correlate with  
 407stomatal size or density parameters, its asymmetry ( $\lambda$  ratio) correlated negatively with SI,  
 408expressing a tendency for the opening delay to be longer with more guard cells per total cells.

409



411Figure 3: Cross correlations between dynamic parameters of the closing sequences ( $\tau$  – response  
 412time,  $\lambda$  – lag time and  $SL_{max}$  – maximum slope) and stomatal parameters (SS -stomatal size, SD -  
 413stomatal density and SI -the stomatal index). Black dots for “Control”, white squares for “Shade”  
 414and white triangles for “Drought” treatment.

## 4164 Discussion

### 4174.1 Treatments impact on steady states values

418 Under the high irradiance conditions of SS1 and SS3, both treatments reduced  $g_s$  and  $A$  compared  
419 to control, where “Drought” had a stronger impact on  $g_s$  and “Shade” a stronger impact on  $A$ . The  
420 latter is probably due to a reduced photosynthetic capacity for the plants of the “Shade” treatment  
421 (confirmed by unpublished data). Stomatal closure under drought is a well studied response  
422 (Turner, 1974; Tardieu and Davies, 1992; Giorio et al., 1999), whereas the stomatal closure under  
423 shade might be due to a  $C_i$  mediated signal to optimize the leaf internal  $CO_2$  concentration (Mott,  
424 1988).

### 4254.2 Impact of the treatments on the dynamic response to irradiance

426 Kirschbaum et al. (1988) proposed a dynamic model in which the response to irradiance was  
427 hypothesized to be composed of three functional steps: first, a biochemical signal that responds  
428 directly to irradiance, then the subsequent variation of osmotic potential causing finally the  
429 movement of water, in/out the guard cells, inducing the actual stomatal movement. From our model  
430 we extracted two parameters ( $\tau$  and  $\lambda$ ) both expressed as time constants, where  $\lambda$  (as a lag time  
431 estimate) could be related to the time needed for the first biochemical signal induction, such as the  
432 phototropin I and II or zeaxanthin (Demming-Adams et al., 1989; Christie, 2007). Further,  $\tau$ ,  
433 describing the steepness of the sigmoidal shape (Fig. 1b), could be related to the response time of  
434 the stomatal movement itself, which might be related to the ion and water fluxes operating during  
435 stomatal movements (Blatt, 2000).

436 Similarly to the steady state parameters, the dynamic response to irradiance has been significantly  
437 changed by both treatments and resulted in contrasting stomatal behaviours in terms of opening and  
438 closing. For the “Control” treatment, the range values of  $\tau$ ,  $\lambda$  and  $SL_{max}$  were comparable to a study  
439 on multiple species (including *Nicotiana tabaccum*) using a similar dynamic model and irradiance  
440 variations (McAusland et al., 2016). This study also showed a strong relationship between  $\tau$  and  
441  $SL_{max}$  across species. The different treatments used in our study allowed a more detailed analysis of  
442 the overall coordination between lag and response times. The “Drought” treatment decreased lag ( $\lambda$ )  
443 and response ( $\tau$ ) times for opening as well as closing, with a stronger impact on closing for  $\lambda$ , but a  
444 stronger impact on opening for  $\tau$ . No impact of drought was visible for  $SL_{max}$  due to a simultaneous  
445 decrease in amplitude. It has been suggested that plants from drier climates or experiencing a  
446 drought stress showed similar faster responses (Vico et al., 2011; Lawson and Blatt, 2014). To the  
447 best of our knowledge, only a few other studies have investigated experimental drought impact on

448the dynamic of stomatal response (Qu et al., 2016; Haworth et al., 2018). Both studies observed  
449faster responses associated to drought during stomatal closing, however, Haworth et al. (2018)  
450found no impact of drought on the opening sequence, which differs from our results. Therefore,  
451literature results as well as our study seem to suggest an increase in stomatal speed under drought,  
452however a conclusion on a differential impact between opening and closing will still need more  
453experimental evidence. The coordinated response of the two time constants towards more rapid  
454stomatal responses (reduced  $\tau$  and  $\lambda$  values) during drought suggest a tighter coupling between  $A_n$   
455and  $g_s$ . This might reduce the loss of water, both at the instantaneous and long-term scale  
456(McAusland et al. 2016) and thereby improve water use efficiency.

457To our knowledge, only few studies have estimated stomatal dynamics on experimental shade or  
458low irradiance growth conditions (Kardiman and Raebild, 2017; Matthews et al., 2018). Our results  
459on  $\tau$  and  $SL_{max}$  for closing tended to be in agreement with these previous studies in which shade  
460grown plants displayed slower stomatal responses to irradiance, however for opening no differences  
461to “Control” was shown. Similarly to the “Drought”, also for the “Shade” treatment, the lag time ( $\lambda$ )  
462showed an acclimation in the opposite direction to a faster response. These results suggest that  
463response ( $\tau$ ) and lag times ( $\lambda$ ) not only acclimated independently to the prevailing environmental  
464treatments, but also that opening and closing mechanisms were not affected similarly by  
465environmental conditions. Such differences between opening and closing response times ( $\tau$ ) might  
466be partly due to the differential ion flux pathways involved in solute uptake and loss involved in  
467stomatal opening and closing, respectively (Blatt, 2000; Shimazaki et al. 2007; Lawson and Blatt  
4682014). Moreover, Haworth et al. (2018) have suggested that the free-ABA content might also have a  
469large influence of the speed of stomatal movements. The acclimations observed for lag time might  
470be more dependent on signalling pathways for irradiance signals (Blatt, 2000), but also via leaf  
471internal  $CO_2$  concentration, modified by the irradiance impact on photosynthesis (Hiyama et al.  
4722017).

473There was a strong asymmetry towards slower stomatal opening in the “Control” treatment for  
474 $SL_{max}$  as well as  $\tau$  and  $\lambda$ . According to Woods and Turner (1971) an asymmetry in this direction  
475might be an adaptation to reduce water loss as a fast stomatal closing allows a tighter coupling  
476between  $A_n$  and  $g_s$  thus reducing excessive loss of water (Tinoco-Ojanguren and Percy, 1992; Ooba  
477and Takahashi, 2003). A slower opening might also limit overshooting situations where stomata  
478continue to open after an increase in irradiance, even when photosynthesis is saturated  
479(McAusland et al. 2016). However, no asymmetry was found for the “Drought” treatment  
480concerning  $SL_{max}$  and  $\tau$ , suggesting a stronger impact of “Drought” on the physiological  
481mechanisms affecting the opening speed compared to “Control”. Whereas for  $\lambda$  the asymmetry was

482even significantly stronger under drought, suggesting that the speed of the biochemical signalling of  
483the irradiance change was more increased for closing, even if also the opening lag time was more  
484rapid compared to control.

485Ooba and Takahashi (2003) suggested that light limited environments would favour a more rapid  
486increase in  $g_s$  as a faster stomatal opening would allow an improved coupling between  $A$  and  $g_s$  and  
487should theoretically increase the overall  $CO_2$  uptake. The experimental “Shade” treatment used here  
488did increase the response time for closing relatively more than for opening, however this was not  
489seen for  $SL_{max}$ , which was more reduced for closing. By dissecting the speed into several parameters  
490as  $\tau$ , the response time (independent of amplitude), the amplitude itself and  $\lambda$ , the lag time, we were  
491able to show that these parameters were affected differently, both “Drought” and “Shade”  
492treatments equilibrated the response times between opening and closing, whereas the asymmetry of  
493the lag time was significantly accentuated by the “Drought”. This could suggest that irradiance  
494response signalling pathways as well as physiological mechanisms relating to stomatal movements  
495might be different between opening and closing and acclimate differently to environmental  
496constraints. However, to substantiate such a hypothesis, more detailed studies are necessary on the  
497molecular level.

#### 4984.3 *Acclimation of stomatal morphology to drought and shade*

499Plants are known to adjust stomatal density, index and size during leaf development to the  
500prevailing environmental conditions (Rawson and Craven, 1975; Carins Murphy et al., 2012; Kalve  
501et al., 2014; McAusland et al., 2016). The decrease of stomatal density (SD) with increasing  
502atmospheric  $CO_2$  concentration is well documented (Woodward, 1987; Pal et al., 2005; Franks and  
503Beerling, 2009); however, there is no clear consensus about the impact of drought on stomatal  
504morphology. Theodorou et al. (2013) observed antagonistic responses of SD among genotypes of  
505grapevine cultivars submitted to drought. Similar contrasting results have been reported in tree  
506(Laajimi et al., 2011) or grass species (Xu and Zhou, 2008). In this study, tobacco plants displayed  
507no acclimation to water deficit in terms of stomatal size, density or index, despite the high intensity  
508of the water stress during the whole growing period, and thus the development of the measured  
509leaves.

510Concerning shade growth conditions, most of the literature suggests a decrease of SD (Gay and  
511Hurd, 1975; Brodribb and Jordan, 2011; Kardiman and Raebild, 2017; Matthews et al., 2018),  
512linked to a lower stomatal index (SI) (Ashton and Berlyn, 1994, Sun et al., 2003; Aasamaa and  
513Aphalo, 2016; Carins-Murphy et al., 2016;). This was also observed here, where the plants grown  
514under shade displayed a significantly reduced SD, linked to a decrease in SI but no change of the



515stomatal size, which corroborates the above cited literature results on woody and herbaceous  
516species other than tobacco.

517

#### 5184.4 Relationship between dynamic parameters and stomatal morphology

519Most of the studies on the relationships between stomatal morphology and dynamics report a faster  
520stomatal response associated with smaller stomata or a higher stomatal density (Hetherington and  
521Woodward 2003; Franks and Farquhar, 2007; Drake et al., 2013; Raven, 2014, Xiong et al., 2017).  
522Nevertheless, other recent studies did not detect any significant correlation between stomatal  
523density, size and the rapidity of response (Haworth et al., 2015; Aasamaa and Aphalo, 2016; Elliot-  
524Kingston et al., 2016). Most of these studies focused on the inter-specific diversity while only one  
525explored within species variation induced by an experimental shade (Aasama and Aphalo, 2016). In  
526all of these studies the rapidity of response was expressed as speed, which is  $g_s$  variation over time.  
527In our study we were able to decompose the speed of stomatal response ( $SL_{max}$ ) into several  
528parameters ( $\tau$  and  $SA_{gs}$ ), and showed that the similar  $SL_{max}$  found in “Control” and “Drought”  
529treatments resulted from significantly different response times ( $\tau$ ) and amplitudes of stomatal  
530responses and therefore very different dynamic responses under these two treatments (Table 2).  
531McAusland et al. (2016) have used a similar model and have found across species with elliptical  
532shaped stomata that pore length correlated with speed ( $SL_{max}$ , for opening only), probably related to  
533the amplitude of the response (strong correlation with steady state  $g_s$ ), but not with the response  
534time ( $\tau$ ). In our study, within one species but across treatments, we did not find any correlation  
535between dynamic parameters and guard cell length, width or surface, similarly to Aasama and  
536Aphalo (2016). This result might be due to the relatively reduced variability of stomatal sizes within  
537species, despite the range of environmental conditions. However, there were significant correlations  
538between stomatal density or index and closing related parameters such as  $SL_{max}$  and  $\tau$ . For the latter  
539this was clearly a co-variation related to the differences among treatments, however for  $SL_{max}$  this  
540was less clear (Fig. 3) and might therefore suggest an effect of the number of stomata on stomatal  
541closing speed, with more stomata resulting in faster dynamics. Such a more rapid stomatal response  
542with higher stomatal density had already been suggested for opening sequences by Violet-Chabrand  
543et al. (2016) using simulations. Finally, the observation of strong differences in stomatal dynamics  
544without (and not related to) a strong variation in stomatal size leads us to the conclusion that within-  
545species, acclimation of stomatal lag and response times involve other mechanisms than stomatal  
546morphology. Such mechanisms could include physiological de-/activation of ion transport in the  
547stomatal guard cells, or a genetic control on the expression of ion transport channels.

#### 5485 Conclusion

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549In this study we highlighted the strong impact of “Drought” and “Shade” treatments on the dynamic  
550response of stomata to variations in irradiance in tobacco plants. The rapidity of response was  
551affected by both treatments but in different ways, where “Drought” reduced both the delay and  
552response times (both faster), “Shade” treatment also reduced the delay but slowed the response  
553time, thus significantly changing the shape and thus the dynamic of the response. Moreover, we  
554showed different stomatal dynamics between closing and opening sequences among treatments,  
555suggesting the existence of different signalling pathways and/or mechanisms involved in the  
556asymmetrical response to irradiance in tobacco plants. However, these tests were only performed  
557with one very large step-change in irradiance. An important perspective would be to confirm the  
558coherence of these results with step-changes of different amplitudes and different starting  
559irradiances.

560The impact of the “Shade” treatment on the stomatal dynamics could be an indication that when  
561introducing such dynamics into canopy scale models, a different parametrization between sun and  
562shade leaves might have to be taken into account. To gain a more mechanistic insight into the  
563acclimation of stomatal dynamics to the growth environment, a more molecular approach would be  
564necessary to observe short-term variations of guard-cell gene expression, ion channel functioning or  
565abundance.

#### 566**Declaration of interest**

567All authors disclose any financial or personal conflict of interest.

568

#### 569**Author contributions**

570TG, CD, OB designed the experiment, CD and JF provided study material and environment, TG  
571and CD conducted the experiment, TG, CD, OB did the data analysis, and TG, CD, OB, JF wrote  
572the manuscript and were involved in the interpretation and critical discussion of the results, OB and  
573JF obtained funding.

574

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