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

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25

26

27Abstract

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

44

45Highlights

- 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 CO₂ 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; Pearcy et al., 2000). Usually stomata respond to low CO₂, 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⁺) 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 CO₂ 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

relationship between stomatal size and closing speed, however the authors suggest a relationship with the atmospheric CO₂ levels during species diversification. Moreover, variations in stomatal size are often negatively correlated to variations in stomatal density (Franks and Beerling, 2009; Doheny-Adam et al., 2012). Also, the relationship between stomatal response times and the shape of the guard cells (Hetherington and Woodward, 2003; Franks and Farquhar, 2007; McAusland et al., 2016) and their patterning (Papanatsiou et al., 2016) have been studied. It has been shown that the stomatal shape (dumbbell or elliptical) might be a determinant driver of stomatal speed as dumbbell-shaped guard cells species tend to display faster responses to environmental fluctuations (Hetherington and Woodward, 2003; MacAusland et al., 2016). Moreover, the stomatal shape and patterning might confer different mechanical advantages via cell osmotic and turgor pressures influencing the rapidity of stomatal response (Franks and Farquhar, 2007). However, other parameters than stomatal morphology, such as variations in ion and water transport within guard cells, are likely to impact the speed of stomatal responses (Lawson and Blatt, 2014).

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

Differences in the speed of stomatal dynamics to a variation in irradiance have been shown among species and within individuals of the same species (Vico et al., 2011; McAusland et al., 2016). Stomatal speed has also been linked to plant functional types and environmental factors (Vico et al., 2011): graminoids tended to display shorter responses than forbs, woody gymnosperms or angiosperms and plants from dryer climates seemed to exhibit faster responses. Moreover, many species 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 ($400\text{--}450\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$) and well-watered conditions. The
185"Shade" treatment was characterized by low irradiance ($40\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$) 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 $30\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ for
188"Control" and $27\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ for "Shade" treatments; 90% of maximum A was reached at about
189 $100\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ for "Control" and at about $990\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ for "Shade"; 90% of maximum g_s for
190"Shade" was reached at $500\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$. 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 (Ψ) 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

Gas exchange was measured using a portable photosynthesis system (LI-COR 6400; LI-COR, Lincoln, NE, USA) equipped with a 2cm² leaf chamber (Li-6400-40). Measured were: net CO₂ assimilation rate (A_n), stomatal conductance for water vapour (g_s) and leaf internal CO₂ concentration (C_i) (see Table 1 for units). All measurements were carried out between 10:00 and 19:00 h (Central European summer time). For each plant measured, gas exchange measurements were performed on the youngest, mature, fully expanded leaf, which had grown under treatment conditions. This leaf has been measured three times at different days and different times during the day. Overall, the measurement of all stomatal response curves has taken 18 days. The environmental parameters inside the chamber were kept constant during the acclimation phase with [CO₂] entering the chamber of 400 $\mu\text{mol mol}^{-1}$, block temperature of 25°C, air flow of 300 $\mu\text{mol min}^{-1}$ and a PPFD of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (red/blue irradiance 90/10%, respectively) until the leaf reached a steady-state of g_s (SS1; Fig. 1a). Then a measurement cycle consisted of two step-changes in irradiance: first I) a single step-change to low irradiance inducing stomatal closure and then II) a single step-change back to the original high irradiance, inducing a stomatal reopening. For the low irradiance step, the PPFD was lowered from 1500 to 100 $\mu\text{mol.m}^{-2} \text{s}^{-1}$ until the plant reached a new steady-state (SS2). After 10 minutes under this new steady-state, the PPFD was set back to its initial setting at 1500 $\mu\text{mol.m}^{-2} \text{s}^{-1}$ and measurements were recorded until a new steady state was reached (SS3). The stomata were considered in steady-state when g_s did not vary more than $\sim 0.005 \text{ mol m}^{-2} \text{s}^{-1}$ during 10min. This resulted in a standard deviation over the 10 minutes of $0.0015 \text{ mol m}^{-2} \text{s}^{-1}$. Data during the response curves were logged every 60sec. “Steady-state” data as mentioned through the manuscript were calculated for SS1, SS2 and SS3 as the mean of 5 points after stabilization of 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 τ (response time) on the
 236 curvature of the sigmoidal model, and c: Simulation of the impact of 600secs steps increasing
 237 values of λ (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. 1cm² 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

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

2.5 Model description

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

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

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

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

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

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

As SS1 and SS3 values for all gas exchange variables were not significantly different (pairwise t-test $p > 0.05$) the mean amplitude between closing and opening in response to the step-change in irradiance were calculated for A_n and g_s as absolute and relative values (SA and RSA, respectively; 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 τ and λ 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 Ψ of tobacco plants, with the highest value
301for the “Shade” treatment (-0.37 ± 0.06 Mpa), without being significantly different from the
302“Control” treatment (-0.51 ± 0.09 Mpa, $p > 0.05$ TukeyHSD), while the “Drought” treatment
303significantly decreased Ψ to -1.46 ± 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 (τ) ranged from ~ 90 s for the
324faster responses to ~ 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 τ values between opening and closing sequence for the “Control”
327treatment, with an asymmetrical response of ~ 2 times slower opening, whereas “Drought” and
328“Shade” did not show a significantly asymmetric responses.

329

Table 1: Means per treatment ($\pm$ standard error of the mean) of net CO₂ assimilation (A_n), stomatal conductance (g_s), internal CO₂ concentration (C_i) at each steady-state reached during the measurement cycle (SS1-SS2-SS3, see Figure 1a for details). The amplitude of variation of the parameters between each steady state (SA and RSA, respectively in absolute values and percentage). For C_i , amplitude of variations was not shown as no differences between the steady states were observed. Different letters show the significant differences among treatments using a Tukey-HSD test. “*” represents the significant differences between two steady states from a paired t-test at $p < 0.05$. There were no significant differences between SS1 and SS3, which were therefore not indicated in the table.

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

339

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

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

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

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

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

361 In Figure 2, the mean of the estimated parameters for each treatment was applied to the sigmoidal
 362 model 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
 365 more rapidly compared to the other two treatments. As the normalized graphs do not depend on the
 366 amplitude, they illustrate the difference between the “Control” and “Drought” treatments in terms of
 367 τ and λ : for a similar overall response time, the “Shade” treatment showed a shorter time lag of the
 368 response.

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

377

3783.4 Stomatal morphology in response of different treatments

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

Significantly lower SD and CD as well as SI values were observed for the “Shade” treatment (Table 3833), whereas no significant differences among treatments were observed for GCW, GSH and SS,

384

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

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

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Table 4. Correlation table of dynamic parameters and the stomatal morphology across treatments. The upper-right triangle displays the p-values (with “***” 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 (Pearson test). With the dynamic parameters: τ , λ , $SL_{\max}$ (where closing slopes are negative), their closing/opening ratio, the absolute amplitude of stomatal conductance response (SA), and the stomatal parameters: GCW (guard cells width), GCL (guard cells length), SS (stomatal size), SD (stomatal density), and SI (the stomatal index). $N=18$ for within dynamic parameter correlations and $N=15$ for correlations with morphology traits, bold r-values are highly significant (***).

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

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3973.5 Cross-correlations between stomatal dynamics and morphology

398The dynamic parameters τ , λ , $SL_{\max}$ and SA displayed significant and high correlations between
399their opening and closing values (Table 4). τ , λ and $SL_{\max}$ also correlated with each other, especially
400within the same irradiance change sequences, except for λ 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 λ and not with τ or $SL_{\max}$.

403There were no correlations between τ , λ , $SL_{\max}$, SA and the stomatal size parameters (GCW, GCL,
404SS). For closure, τ and $SL_{\max}$ showed small negative correlations with SD and SI (Fig. 3),
405associating more stomata with faster responses. The τ relationships were more clearly driven by the
406treatments differences than the $SL_{\max}$ relationships (Fig. 3). Whereas λ did not correlate with
407stomatal size or density parameters, its asymmetry (λ 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 (τ – response
412time, λ – 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 (τ and λ) both expressed as time constants, where λ (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, τ ,
 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 τ , λ 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 τ 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 (λ)
 443 and response (τ) times for opening as well as closing, with a stronger impact on closing for λ , but a
 444 stronger impact on opening for τ . 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

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

To our knowledge, only few studies have estimated stomatal dynamics on experimental shade or low irradiance growth conditions (Kardiman and Raebild, 2017; Matthews et al., 2018). Our results on τ and SL_{max} for closing tended to be in agreement with these previous studies in which shade grown plants displayed slower stomatal responses to irradiance, however for opening no differences to “Control” was shown. Similarly to the “Drought”, also for the “Shade” treatment, the lag time (λ) showed an acclimation in the opposite direction to a faster response. These results suggest that response (τ) and lag times (λ) not only acclimated independently to the prevailing environmental treatments, but also that opening and closing mechanisms were not affected similarly by environmental conditions. Such differences between opening and closing response times (τ) might be partly due to the differential ion flux pathways involved in solute uptake and loss involved in stomatal opening and closing, respectively (Blatt, 2000; Shimazaki et al. 2007; Lawson and Blatt 2014). Moreover, Haworth et al. (2018) have suggested that the free-ABA content might also have a large influence of the speed of stomatal movements. The acclimations observed for lag time might be more dependent on signalling pathways for irradiance signals (Blatt, 2000), but also via leaf internal CO_2 concentration, modified by the irradiance impact on photosynthesis (Hiyama et al. 2017).

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

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

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

4.3 *Acclimation of stomatal morphology to drought and shade*

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

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

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

517

5184.4 Relationship between dynamic parameters and stomatal morphology

Most of the studies on the relationships between stomatal morphology and dynamics report a faster stomatal response associated with smaller stomata or a higher stomatal density (Hetherington and Woodward 2003; Franks and Farquhar, 2007; Drake et al., 2013; Raven, 2014, Xiong et al., 2017). Nevertheless, other recent studies did not detect any significant correlation between stomatal density, size and the rapidity of response (Haworth et al., 2015; Aasamaa and Aphalo, 2016; Elliot-Kingston et al., 2016). Most of these studies focused on the inter-specific diversity while only one explored within species variation induced by an experimental shade (Aasama and Aphalo, 2016). In all of these studies the rapidity of response was expressed as speed, which is g_s variation over time. In our study we were able to decompose the speed of stomatal response (SL_{max}) into several parameters (τ and SA_{gs}), and showed that the similar SL_{max} found in “Control” and “Drought” treatments resulted from significantly different response times (τ) and amplitudes of stomatal responses and therefore very different dynamic responses under these two treatments (Table 2). McAusland et al. (2016) have used a similar model and have found across species with elliptical shaped stomata that pore length correlated with speed (SL_{max} , for opening only), probably related to the amplitude of the response (strong correlation with steady state g_s), but not with the response time (τ). In our study, within one species but across treatments, we did not find any correlation between dynamic parameters and guard cell length, width or surface, similarly to Aasama and Aphalo (2016). This result might be due to the relatively reduced variability of stomatal sizes within species, despite the range of environmental conditions. However, there were significant correlations between stomatal density or index and closing related parameters such as SL_{max} and τ . For the latter this was clearly a co-variation related to the differences among treatments, however for SL_{max} this was less clear (Fig. 3) and might therefore suggest an effect of the number of stomata on stomatal closing speed, with more stomata resulting in faster dynamics. Such a more rapid stomatal response with higher stomatal density had already been suggested for opening sequences by Violet-Chabrand et al. (2016) using simulations. Finally, the observation of strong differences in stomatal dynamics without (and not related to) a strong variation in stomatal size leads us to the conclusion that within-species, acclimation of stomatal lag and response times involve other mechanisms than stomatal morphology. Such mechanisms could include physiological de-/activation of ion transport in the stomatal 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
559irradiance.

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.

566Declaration of interest

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

568

569Author 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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