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

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27**Abstract**

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

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

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50 **1 Introduction**

51 Stomatal morphology and their movements (opening and closing) are key components controlling 52 exchange of water vapour and CO_2 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 CO_2, 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 (Vialet- 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 CO_2 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

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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 has 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

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118relationship between stomatal size and closing speed, however the authors suggest a relationship 119with the atmospheric CO_2 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 Woodwaard, 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 Woodwaard, 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_s 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

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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.

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175## 2 Material and methods

176*2.1 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 (400-450 µmol photons m^{-2} s^{-1}) and well-watered conditions. The 185"Shade" treatment was characterized by low irradiance (40 µmol photons m^{-2} 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 30µmol m^{-2} s^{-1} for 188"Control" and 27 µmol m^{-2} s^{-1} for "Shade" treatments; 90% of maximum A was reached at about 1891100µmol m^{-2} s^{-1} for "Control" and at about 990µmol m^{-2} s^{-1} for "Shade"; 90% of maximum g_s for 190"Shade" was reached at 500µmol m^{-2} 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 25ºC, 199air relative humidity 50-60% and a photoperiod of 12h/12h (8:00-20:00).

200*2.2 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.

206*2.3 Gas exchanges measurements*

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207Gas exchange was measured using a portable photosynthesis system (LI-COR 6400; LI-COR, 208Lincoln, NE, USA) equipped with a 2cm² leaf chamber (Li-6400-40). Measured were: net CO_2 209assimilation rate (A_n), stomatal conductance for water vapour (g_s) and leaf internal CO_2 210concentration (C_i) (see Table 1 for units). All measurements were carried out between 10:00 and 21119:00 h (Central European summer time). For each plant measured, gas exchange measurements 212were performed on the youngest, mature, fully expanded leaf, which had grown under treatment 213conditions. This leaf has been measured three times at different days and different times during the 214day. Overall, the measurement of all stomatal response curves has taken 18 days. The 215environmental parameters inside the chamber were kept constant during the acclimation phase with 216[CO_2] entering the chamber of 400 µmol mol^{-1}, block temperature of 25°C, air flow of 300 µmol 217min^{-1} and a PPFD of 1500 µmol m^{-2} s^{-1} (red/blue irradiance 90/10%, respectively) until the leaf 218reached a steady-state of g_s (SS1; Fig. 1a). Then a measurement cycle consisted of two step-changes 219in irradiance: first I) a single step-change to low irradiance inducing stomatal closure and then II) a 220single step-change back to the original high irradiance, inducing a stomatal reopening. For the low 221irradiance step, the PPFD was lowered from 1500 to 100 µmol.m^{-2} s^{-1} until the plant reached a new 222steady-state (SS2). After 10 minutes under this new steady-state, the PPFD was set back to its initial 223setting at 1500 µmol.m^{-2} 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 ~0.005 mol m^{-2} 225s^{-1} during 10min. This resulted in a standard deviation over the 10 minutes of 0.0015 mol m^{-2} s^{-1}. 226Data during the response curves were logged every 60sec. “Steady-state” data as mentioned through 227the manuscript were calculated for SS1, SS2 and SS3 as the mean of 5 points after stabilization of 228g_s, (Fig. 1a).

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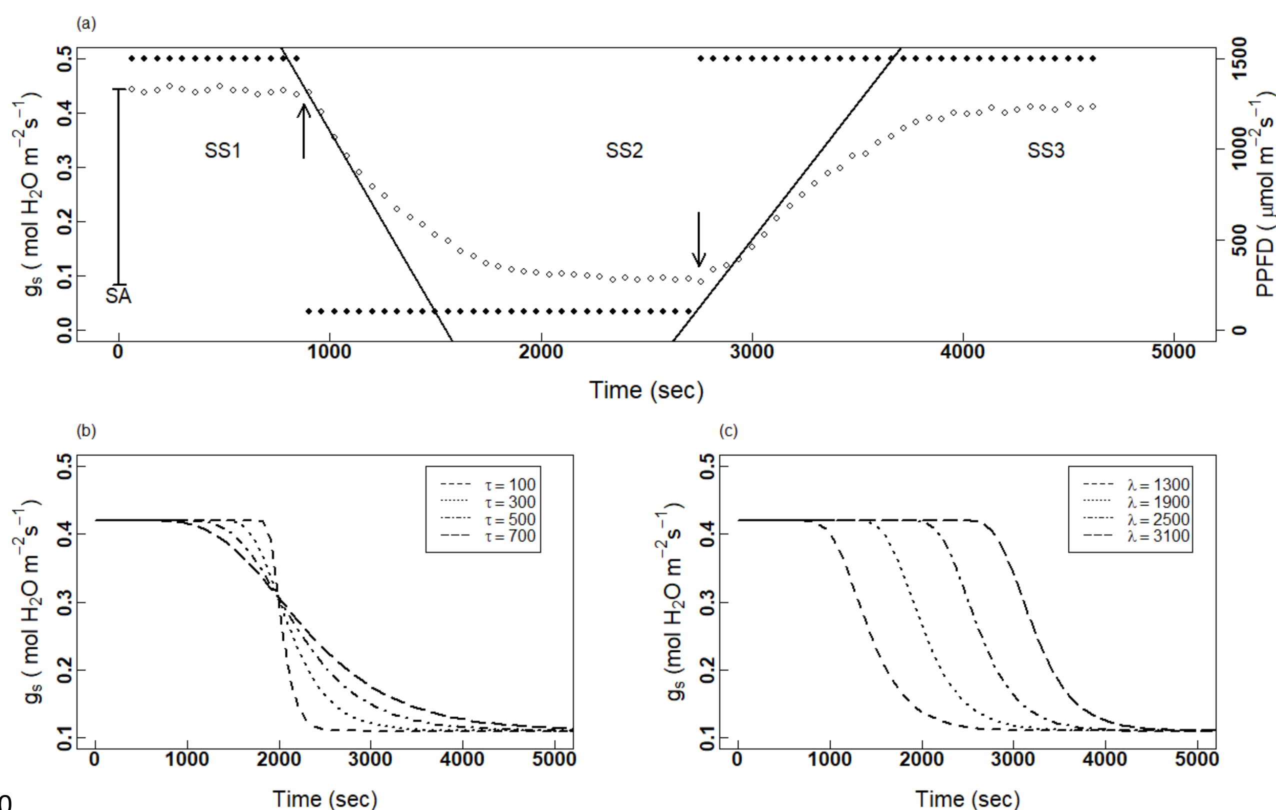

230

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

238

239*2.4 Stomata morphology*

240At the end of the experiment one leaf was sampled from each of five plants per treatment to 241determine the stomatal morphology of leaves on the abaxial and adaxial faces. Specifically, the 242following parameters were measured : the stomatal density (SD), epidermal cell density (CD), 243stomatal index (SI) defined as SD/(SD+CD), length of the stomatal guard cell complex (GCL), 244guard cells width (GCW), stomatal surface (SS) defined as an ellipse area: π*(GCL/2)*(GCW/2) 245and guard cell shape (GSH) defined as the ratio GCL/GCW. 1cm² portions of the leaves were 246collected and nail polish imprints of both leaf surfaces were taken using adhesive film and applied 247on microscope slides for analysis. Stomata and epidermis cells were counted in the obtained images

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

250*2.5 Model description*

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

254$$gs = r0 + (G - r0) * \exp\left(-\exp\left(\frac{\lambda - t}{\tau}\right)\right)$$

255where g_s is the fitted stomatal conductance, r0 is the starting value of the stomatal conductance (first 256steady-state obtained after the plant acclimation to the environmental conditions inside the Licor 257chamber, g_{min} or g_{max}), G the ending value of stomatal conductance (second steady-state reached 258after the full stomatal response to the irradiance change, g_{max} or g_{min}), λ is the lag time of the 259stomatal response (time needed to reach the inflection point of the curve from the moment of the 260irradiance change in each curve), and τ the response time. Compared to the sigmoidal equation used 261by Vialet-Chabrand et al. (2013), here, λ is mathematically independent from τ (see Fig. 1 b and c). 262From these parameters, the maximum slope (SL_{max}) as estimator of the speed of the stomatal 263response, can be calculated as:

264$$SLmax = (1/\tau) * (G - r0)/e$$

265Where (G-r0) represents the amplitude of the stomatal response (SA) and e the Euler constant. 266Increasing values of τ will affect the curvature of the stomatal response, the smaller a τ value is, the 267stronger the curvature (Fig. 1b) and the higher SL_{max} will be, so the more rapidly g_s will 268increase/decrease.

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

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

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

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

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

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

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

286

287 *2.6 Statistical analysis*

288 All statistical analyses were performed with R (Team RC, 2015). Treatment effects were analysed
289 as 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-
291 HSD (package R, "agricolae") was used to estimate significance of inter-group differences.
292 Comparisons among steady-state gas exchange variables as well as of closing versus opening were
293 done using pairwise t-tests. For all stomatal morphology traits, mean values of all images taken on
294 the adaxial and abaxial surfaces were used for ANOVA and correlations with gas exchange results
295 were based on a mean of the 3 repetitions per leaf. Correlations were estimated using the Pearson
296 method and p-values were adjusted for multiple comparisons using the "p.adjust" function with the
297 "FDR" method.

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298 **3 Results**

299 *3.1 Steady-state gas exchange parameters under three different growth conditions*

300 The different growth conditions significantly affected Ψ of tobacco plants, with the highest value 301 for 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 303 significantly decreased Ψ to –1.46±0.18 Mpa (p>0.05 TukeyHSD). A_n values measured under 304 saturating 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 306 among the treatments, showing the highest values in "Control" and the lowest in "Drought". These 307 strong differences for A_n and g_s were associated with significantly different C_i among treatments, 308 with the highest C_i in the "Shade" and the lowest under "Drought". Decreasing irradiance from high 309 to low intensity (SS1 to SS2, see Fig. 1), A_n and g_s decreased drastically as expected (Table 1), but 310 not C_i, for which no significant differences were detected among the three steady-states, within each 311 treatment.

312 *3.2 Amplitude of response under changing measuring irradiance*

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

322 *3.3 Dynamic parameters of stomatal response under irradiance changes*

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

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330 Table 1: Means per treatment (± standard error of the mean) of net CO_2 assimilation (A_n), stomatal 331 conductance (g_s), internal CO_2 concentration (C_i) at each steady-state reached during the 332 measurement cycle (SS1-SS2-SS3, see Figure 1a for details). The amplitude of variation of the 333 parameters between each steady state (SA and RSA, respectively in absolute values and 334 percentage). For C_i, amplitude of variations was not shown as no differences between the steady 335 states were observed. Different letters show the significant differences among treatments using a 336 Tukey-HSD test. "*" represents the significant differences between two steady states from a paired 337 t-test at $p<0.05$. There were no significant differences between SS1 and SS3, which were therefore 338 not indicated in the table.

Treatments		SS1		SS2		SS3	SA	RSA (%)
Control	A_n (μmol m^{-2} 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 (mol m^{-2} 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	Ci (μmol 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

340 Table 2: Dynamic parameters for the opening and closing sequences, with τ the response time, λ the 341 delay of stomatal response, τ/λ their ratio and SL_{max} the maximal slope of the response. Different 342 letters show the significant differences between treatments from an ANOVA model including 343 treatment effects followed by a post-hoc Tukey test. The number of analysed response curves are in 344 parentheses, with a mean of 2.7 repetitions per leaf. * show the significant differences between 345 closing and opening from a paired t-test (P-values < 0.05), so a significant difference indicates an 346 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} (mol m^{-2} s^{-2} x 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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347

348The lag time of the response (λ) showed a different pattern compared to τ. During the closing 349sequence, "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 351significantly higher for the opening than for the closing sequence (Table 2), with the strongest λ 352asymmetry for the "Drought" treatment while the ratios were similar between "Shade" and 353"Control" treatments.

354For the "Control" treatments, λ and τ 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 λ, whereas 357the "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 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 363g_{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 367τ and λ : for a similar overall response time, the "Shade" treatment showed a shorter time lag of the 368response.

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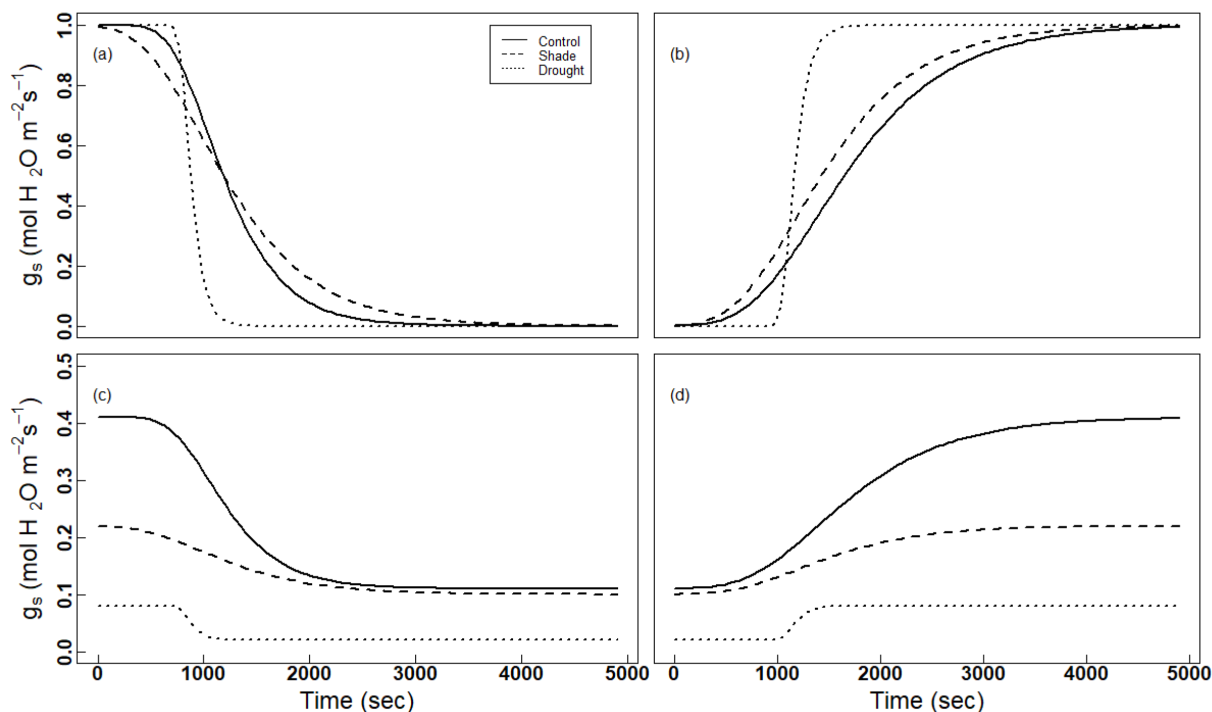

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371Figure 2: Fitted stomatal dynamics induced by changes in light intensity in closing (a, c) and 372opening (b, d) sequences. Plain lines are for "Control", simple dashed lines for "Shade" and dotted 373lines for "Drought" treatment. Figures (a) and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

377

378*3.4 Stomatal morphology in response of different treatments*

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

382Significantly 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,

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385Table 3: Stomatal morphology means for treatments (± standard error of the mean). GCL: guard cell 386length (µm), GCW: pore width (µm), SS: stomatal surface (µm²), GSH: stomatal shape 387(GCL/GCW), SD: stomatal density (mm^{-2}), CD: epidermis cell density (mm^{-2}), SI: stomatal index 388(SD/SD+CD). Different letters show the significant differences between the treatments (Tukey-389HSD).

Treatment	Trait	Mean±SE (N)
Control	GCL (µm)	33.1 ± 1.2^{a} (5)
Drought		35.1 ± 1.7^{a} (5)
Shade		34.0 ± 0.5^{a} (5)
Control	GCW (µm)	26.7 ± 0.8^{a} (5)
Drought		26.4 ± 0.8^{a} (5)
Shade		24.1 ± 0.3^{a} (5)
Control	SS (µm²)	695 ± 55.2^{a} (5)
Drought		731 ± 73^{a} (5)
Shade		666 ± 22^{a} (5)
Control	GSH	1.24 ± 0.02^{b} (5)
Drought		1.33 ± 0.03^{ab} (5)
Shade		1.41 ± 0.02^{a} (5)
Control	SD (mm^{-2})	126 ± 7^{a} (5)
Drought		131 ± 12^{a} (5)
Shade		58 ± 8^{b} (5)
Control	CD (mm^{-2})	478 ± 15^{a} (5)
Drought		544 ± 73^{a} (5)
Shade		318 ± 25^{b} (5)
Control	SI	0.2 ± 0.01^{a} (5)
Drought		0.19 ± 0.01^{a} (5)
Shade		0.15 ± 0.01^{b} (5)

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391Table 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: τ, λ, SL_{max} (where closing slopes are negative), their closing/opening ratio, the absolute amplitude of 394stomatal 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 396r-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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397 *3.5 Cross-correlations between stomatal dynamics and morphology*

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

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

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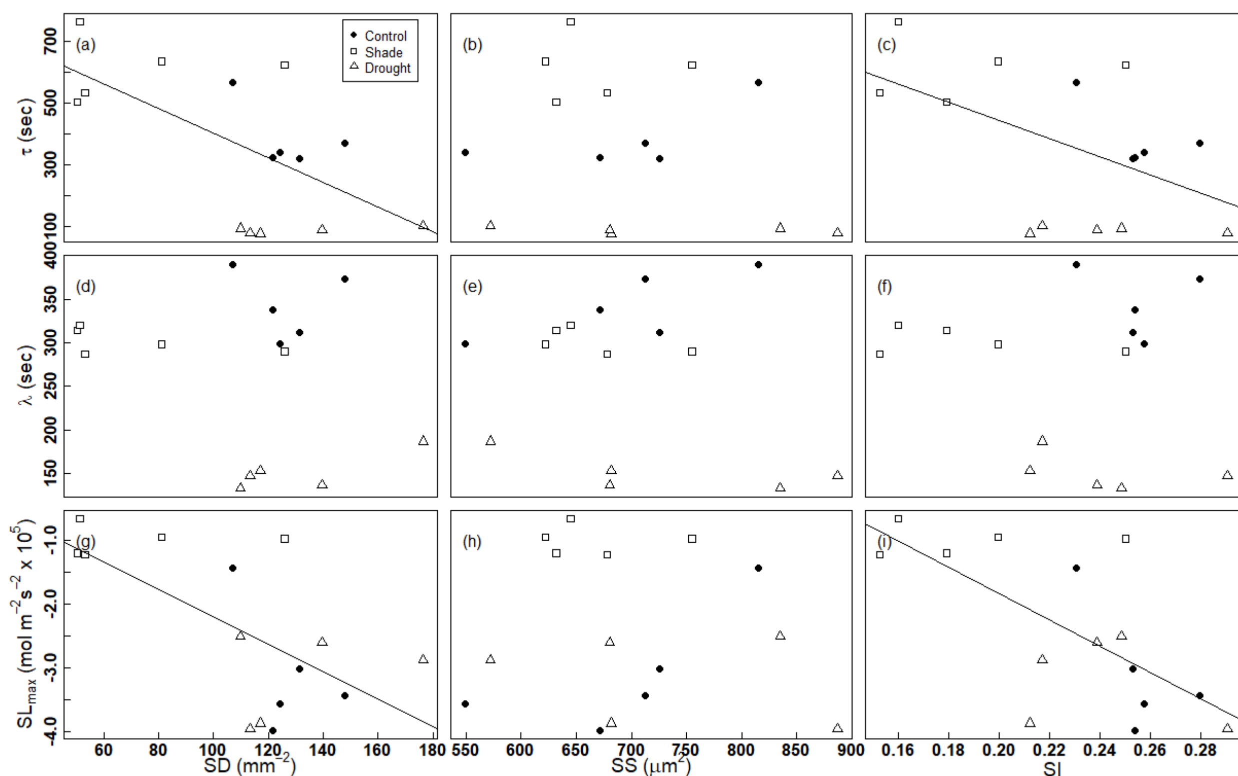

411 Figure 3: Cross correlations between dynamic parameters of the closing sequences (τ – response
412 time, λ – lag time and SL_{max} – maximum slope) and stomatal parameters (SS -stomatal size, SD -
413 stomatal density and SI -the stomatal index). Black dots for “Control”, white squares for “Shade”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

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415

416 4 Discussion

417 *4.1 Treatments impact on steady states values*

418 Under the high irradiance conditions of SS1 and SS3, both treatments reduced gs and A compared 419 to control, where "Drought" had a stronger impact on gs 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).

425 *4.2 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

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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 τ and λ 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 τ 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 (λ) 462showed an acclimation in the opposite direction to a faster response. These results suggest that 463response (τ) and lag times (λ) 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 (τ) 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 474SL_{max} as well as τ and λ. 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 Pearcy, 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(MacAusland et al. 2016). However, no asymmetry was found for the "Drought" treatment 480concerning SL_{max} and τ, suggesting a stronger impact of "Drought" on the physiological 481mechanisms affecting the opening speed compared to "Control". Whereas for λ the asymmetry was

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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 τ, the response time (independent of amplitude), the amplitude itself and λ, 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.

498*4.3 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

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

517
518*4.4 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 (τ and SA_{gs}), and showed that the similar SL_{max} found in "Control" and "Drought"
529treatments resulted from significantly different response times (τ) 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 (τ). 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 τ. 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 Vialet-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.

548**5 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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