RAFAEL SILVA FREITAS

STOMATAL RESPONSE TIME TO DYNAMIC CHANGES IN LIGHT IS ESSENTIALLY UNAFFECTED BY GROWTH LIGH FOR THREE WOODY SPECIES

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do programa de Pós-Graduação em Fisiologia Vegetal, para obteção do título de *Magister Scientiae*.

Orientador: Fábio Murilo DaMatta
Coorientadora: Amanda Ávila Cardoso

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APROVADA: 27 de julho de 2021

Assentimento:

Rafael Silva Freitas
Autor

Fábio Murilo DaMatta
Orientador

All that is solid melts into air.

Delight in the Lord and he will fulfill your heart's desires.

To John and Mary with love.

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Eu sou grato a todos aqueles que me ajudaram antes, durante e depois da consolidação deste trabalho, vocês não imaginam o quão importante foi a ajuda de vocês para a finalização desse ciclo.

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ABSTRACT

FREITAS, Rafael Silva, M.Sc., Universidade Federal de Viçosa, July, 2021. **Stomatal response time to light dynamic changes is essentially unaffected by growth light for three woody species**. Adviser: Fábio Murilo DaMatta. Co-adviser: Amanda Ávila Cardoso.

Stomata are small epidermal pores responsible for the strict control of the amount of CO_2 that diffuses into the leaves while controlling the amount of water vapor lost by them to the atmosphere. The time required for the stomatal valve opening and closing is coordinated with an optimized hydraulic supply and strongly responds to the surrounding environment. Here, we demonstrate that intense shading conditions promote high levels of plasticity in plants of *Podocarpus macrophyllus*, *Eucalyptus urophylla*, and *Capsicum chinense*, in a series of hydraulic, anatomical and gas exchange traits, parameters that have been associated with optimized stomatal kinetics. The high levels of plasticity expressed in the species that we observed (in leaf hydraulic conductance values as well as stomatal size, density, and length) did not necessarily translate into changes in stomatal kinetics, here assessed as the time required to change the initial stomatal conductance in 90%. Our findings demonstrate that, although these parameters are mechanistically involved in stomatal functioning and exhibit profound plastic behavior, a number of other factors that probably are insensible to light supply also dictate this process, rendering the stomatal response time to dynamic changes in light essentially unaffected by growth light conditions.

Keywords: Hydraulic conductance. Irradiance. Light acclimation. Leaf Gas Exchange. Stomatal density.

RESUMO

FREITAS, Rafael Silva, M.Sc., Universidade Federal de Viçosa, julho de 2021. **O tempo de resposta estomática a mudanças dinâmicas na luz não é essencialmente afetado pela luz de crescimento em três espécies lenhosas**. Orientador: Fábio Murilo DaMatta. Coorientadora: Amanda Ávila Cardoso.

Os estômatos são pequenos poros epidérmicos responsáveis pelo rígido controle da entrada de CO_2 que se difunde para o interior das células, ao passo que controlam a quantidade de vapor d'água perdido pelas folhas para a atmosfera. O tempo necessário para a abertura e o fechamento das válvulas estomáticas é coordenado com um sistema de condutos hidráulicos otimizado e responde fortemente ao ambiente adjacente. Neste estudo, foi demostrado que condições de sombreamento intenso promovem grande plasticidade em plantas de *Podocarpus macrophyllus*, *Eucalyptus urophylla* e *Capsicum chinense*, plasticidade esta altamente pronunciada em uma série de características (associadas a uma cinética estomática otimizada) hidráulicas, anatômicas e de trocas gasosas. O alto grau de plasticidade expresso nas espécies, tanto em valores de condutância hidráulica foliar, e tamanho, densidade e comprimento estomático, não necessariamente se traduziram em mudanças na cinética estomática, aqui analisada como tempos necessários para alteração (aumento ou diminuição, dependendo da alteração na irradiância) em 90% da condutância estomática inicial. Dessa forma, os presentes resultados sugerem que, embora os parâmetros ora avaliados sejam mecanisticamente associados ao funcionamento estomático e exibam forte comportamento plástico, uma série de outros fatores, provavelmente insensíveis ao suprimento de luz, também ditam a cinética dos movimentos estomáticos, fazendo com que o tempo de resposta estomática a alterações dinâmicas na luz seja muito pouco afetado pela condição lumínica de crescimento.

Palavras-chave: Condutância hidráulica foliar. Densidade estomática. Irradiância. Sombreamento.

LIST OF FIGURES

LIST OF ACRONYMS AND ABBREVIATIONS

C. chinense	*Capsicum chinense*
A	Net rate of photosynthesis
C	Leaf hydraulic capacitance
g_s	Stomatal conductance
E	Transpiration rate
E. urophylla	*Eucalyptus urophylla*
FAA_{70}	Formaldehyde, acetic acid and 70% ethanol
FOV	Fields of vision
IRGA	Infrared gas analyzer
K_{leaf}	Leaf hydraulic conductance
LA	Leaf area
P. macrophyllus	*Podocarpus macrophyllus*
PPFD	Photosynthetic photon flux density
SD	Stomatal density
SI	Stomatal index
SL	Stomatal lenght
SLA	Specific leaf area
SS	Stomatal Size
T_{90}	Time taken to reach 90%
VD	Vein density
VPD	Vapor pressure deficit
Ψ_w	Water potential

CONTENT

Introduction

Stomata are tiny pores surrounded by a pair of specialized guard cells that actively regulate the majority of all gaseous diffusion between the leaf interior and the surrounding atmosphere and vice-versa (Franks & Farquhar, 2007; Brodribb *et al.*, 2020). The turgor pressure of guard cells adjusts to both internal and external environmental stimuli, ultimately determining the stomatal pore size and thus the foliar porosity to water and CO_2 exchange (Brodribb & McAdam, 2017; Matthews & Lawson, 2019). Amongst all environmental cues regulating stomatal aperture, light appears as the most pervasive one, with a step increase in irradiance inducing stomatal opening and a step decrease causing stomata to close in nearly all vascular land plants (Deans *et al.*, 2018). Such light-induced changes in stomatal aperture are underpinned by complex signaling mechanisms combining blue light-specific and photosynthesis-mediated pathways (Shimazaki *et al.*, 2007; Baroli *et al.*, 2008; Inoue & Kinoshita, 2017).

In natural environments, the incident light irradiance on leaves is not steady, constantly changing as clouds pass and higher leaves move shading different lower leaves resulting in sun and shade flecks (Woods & Turner, 1971). This naturally fluctuating light condition causes stomata and photosynthesis to continually respond to changes in solar radiation, with much faster photosynthetic than stomatal responses (Vico *et al.*, 2011; Lawson & Blatt, 2014; Deans *et al.*, 2018). Therefore, following an increase in irradiance, leaves transiently miss out on potential increments in photosynthesis rates due to slow stomatal opening, while after a decrease in irradiance, leaves continue to lose water due to the slow stomatal closure without maintaining high rates of photosynthesis (Vico *et al.*, 2011; Lawson & Blatt, 2014; Deans *et al.*, 2018). These events highlight the importance of faster stomatal responses to dynamic changes in light to both minimize unnecessary water loss as stomata close and maximize photosynthesis as stomata open (Vico *et al.*, 2011; Lawson & Blatt, 2014).

Across different plant species, a significant variation in the rapidity of stomatal responses to fluctuating light conditions is known to exist (Drake *et al.*, 2013; Elliott-Kingston *et al.*, 2016; Deans *et al.*, 2018; Kardiman & Ræbild, 2018), but the mechanisms controlling stomatal speed are far from being understood (Lawson & Blatt, 2014). Evidence from studies comparing several species correlates lower stomatal size (SS) with faster stomatal responses likely due to their greater membrane surface area to volume ratio (Hetherington & Woodward, 2003; Franks & Beerling,

2009; Drake *et al.*, 2013; Kardiman & Ræbild, 2018). This hypothesis has been drawn based on the assumption that transport activity of guard cells, another important player likely controlling the speed of stomatal responses, remains constant on a unit-surface-area basis. Studies directly examining specific transport activities, however, indicate a substantial variation among species independently of surface area (Chen *et al.*, 2012; Eisenach *et al.*, 2012, 2014), which suggests that *SS* might be of secondary importance to the speed of stomatal movements.

Besides stomatal morphology and physiology, the ecological niches have also been associated with the speed of stomatal responses to light (Woods & Turner, 1971; Deans *et al.*, 2018; Kardiman & Ræbild, 2018). Shade-tolerant species, for instance, have long been suggested to display faster stomatal opening times in response to light, a strategy to better utilize light flecks of short duration inside the canopy (Woods & Turner, 1971; Knapp & Smith, 1987). Despite several studies comparing the rapidity of stomatal responses to changing light across species over the past decades, studies assessing the plasticity of stomatal dynamics (i.e. speed or time for stomatal response) in response to growth environmental conditions are much more recent and far less common (Gerardin *et al.*, 2018; Kardiman & Ræbild, 2018; Matthews *et al.*, 2018). Results from these studies range from limited plasticity of stomatal dynamics in response to light (Kardiman & Ræbild, 2018) to varying degrees of plasticity in the rapidity and magnitude of responses (Gerardin *et al.*, 2018; Kardiman & Ræbild, 2018; Matthews *et al.*, 2018), suggesting a species-specific mechanism, yet not fully understood.

This study investigates the plasticity in stomatal dynamics during step increases and decreases in photon irradiance across woody species grown under contrasting light regimes (sun and shade), a condition known to profoundly change leaf anatomy and function, including stomatal traits and leaf hydraulics (Scoffoni *et al.*, 2008; Carins Murphy *et al.*, 2012, 2016; Martins *et al.*, 2014; Rodríguez-López *et al.*, 2014). Three woody species, including a conifer [*Podocarpus macrophyllus* (Thunb.) D. Don (Podocarpaceae)] and two angiosperms [*Eucalyptus urophylla* S. T. Blake (Myrtaceae) and *Capsicum chinense* Jacq. (Solanaceae)], spanning a wide phylogenetic basis, were selected for this study based on their ability to grow under contrasting light irradiances. Specifically, we aimed at understanding, whether potential light-induced changes in stomatal morphology [especially stomatal density (SD), stomatal length (SL), and stomatal size (SS)] and in leaf hydraulic conductance (K_{leaf}) [which is known to be regulated by growth and dynamic light

(Scoffoni *et al.*, 2008; Carins Murphy *et al.*, 2016) and defines stomatal conductances in leaves (Brodribb *et al.*, 2007)] would result in stomatal dynamic plasticity.

Materials and Methods

Plant material and experimental conditions

Plants of *C. chinense* were grown from seeds, while saplings of *P. macrophyllus* and *E. urophylla* were obtained from the nursery of the Department of Forest Engineering at the Universidade Federal de Viçosa, Viçosa (20°45′ S, 42°54′ W, 650 m above sea level), Brazil. One-month-old seedlings of *C. chinense* and six-month-old saplings of *P. macrophyllus* and *E. urophylla* were transplanted into 20-L pots filled with a potting mix (3:3:1 mix of topsoil, washed river sand and the commercial potting soil Tropstrato HT Hortaliças), supplemented with a slow-release fertilizer. Plants were next moved to the gardens of the Universidade Federal de Viçosa and randomly positioned, where they remained from June 2020 to March 2021. Four plants of each species were grown under either full sunlight (sun-acclimated) or 90% shade (shade-acclimated) using neutral density black nylon nettings, which have been shown not to affect the light quality (Rodríguez-López et al., 2014). The maximum photosynthetic photon flux density (PPFD) of both light conditions was weekly measured between 11:00 and 13:00 h (solar time) using two identical line quantum sensors (LI-191R; LI-COR, Lincoln, NE, USA) (Supplementary Figure 1A). Plants grown at full sunlight received a maximum PPFD of c. 2050 µmol photons m^{-2} s^{-1}, while plants under shade received a maximum PPFD of c. 200 photons µmol m^{-2} s^{-1}, as measured at midday. All plants were daily irrigated and to make sure both sun- and shade-acclimated plants were well-watered, we assessed the predawn and midday water potentials of all plants during three cloudless days using a pressure chamber (Model 1000, PMS Instruments, Albany, OR, USA) (Supplementary Table 1). Temperature and relative humidity at which plants were grown were also weekly assessed and both sun and shade plants were exposed to similar conditions (Supplementary Figure 1B). All measurements described below were performed using completely expanded leaves that were developed entirely either at full sunlight or at shade, according to the treatments.

Leaf morphology and anatomy

A leaf sample from each plant was scanned using a flatbed scanner to measure leaf area using Image Pro-Plus (Media Cybernetics, Rockville, MD, USA), and then further utilized to assess

anatomical traits. Specific leaf area was obtained utilizing additional leaves from which leaf area and leaf dry mass were obtained. For anatomy, leaves were fixed in FAA70 (formaldehyde, acetic acid and 70% ethanol) during 48 h and then stored in 70% aqueous ethanol. Paradermal sections were obtained by a clearing and staining protocol (Strittmatter, 1973) utilizing 2 cm^2 sections selected from the central regions of each leaf blade, taking care to avoid major veins. Briefly, sections were immersed in 10% NaOH (w v^{-1}) and bleached in 20% common house bleach solution (w v^{-1}). A stain solution composed by Safranin-O and Crystal violet was used in an oven at 60°C to stain lignin-rich tissues. Sections were then dehydrated using graded ethanol series (50, 60, 70, 80, 90 and 100%) and immersed in ethanol-xylol series (3:1, 1:1 and 1:3; v v^{-1}). Sections were photographed using a light microscope (AX70 TRF, Olympus Optical, Tokyo, Japan) coupled with a digital camera (Zeiss AxioCam HRc, Göttingen, Germany). Five to ten fields of view (FOV) at 20× magnification for *SD*, *SL* and *SS* were photographed and images were analyzed using Image Pro-Plus. The *SD* was quantified as the number of stomata per mm^2 of leaf area, *SL* as the maximum length of stomata measured at the center, and *SS* as *SL* multiplied by stomatal width of the closed guard cell pair (Franks and Beerling, 2009). *SL* and *SD* were further calculated as the mean of all stomata of all images per leaf sample.

Leaf gas exchange and hydraulic conductance

Gas exchange was measured *in situ* from leaves from the uppermost parts of the canopy using a portable open-system gas exchange analyzer (LI-6400XT, LI-COR, NE, USA). Measurements of the net rate of photosynthesis (A) and stomatal conductance to water vapor (g_s) were performed in all plants on three different cloudless days between 09:00 and 11:00 h (solar time) when gas exchange is expected to be at maximum, and the data of each plant are presented as an average of the three measurement days. Conditions within the cuvette were adjusted to either 1000 or 100 μmol photons m^{-2} s^{-1} (which approximately corresponded to natural light intercepted by sun- and shade-acclimated leaves, respectively) and temperature at 25°C, while natural CO_2 (c. 410 ppm) and vapor pressure deficit (VPD) (which ranged from 0.5 to 1.5 kPa) were utilized.

The K_{leaf} was measured using the same leaves latter utilized for leaf area and anatomy using the evaporative flux method and flowmeter (Sack *et al.*, 2002; Brodribb & Holbrook, 2006). Leaves were excised under water, immediately attached to a custom-build flow meter and placed in conditions to induce increased transpiration rates (i.e. under a light source and above a large

cooler). Different sun and shade-acclimated leaves were exposed to either 100 µmol photon m^{-2} s^{-1} or 1000 µmol photon m^{-2} s^{-1}. Once leaves reached a steady-state transpirational rate (less than 10% variation over 5 min), they were detached and had their water potential (Ψ_w) measured with a pressure chamber. The K_{leaf} was then calculated by dividing the transpirational rates at steady-state by the Ψ_w. Values were standardized for leaf area and for the viscosity of water at 25°C, using an empirical function based on data from Korson et al. (1969).

Stomatal dynamics measurements

Prior to analyses of stomatal responses to light, plants were transferred to the laboratory and maintained overnight in the dark. During the next morning, leaves were enclosed in a conifer chamber (6400-22L, LI-COR, NE, USA) connected to a portable open-flow gas exchange system (LI-6400XT, LI-COR, NE, USA) and then subjected to dark and natural conditions of temperature (c. 28 ± 0.7°C), CO_2 (c. 410 ppm) and vapor pressure deficit (VPD) (c. 1.4 ± 0.1 kPa). Leaves were kept under these conditions until steady-state g_s and A was reached, after which gas exchange parameters were logged every 30 s. After 10 min of steady-state measurements under darkness, irradiance was increased to 100 µmol photon m^{-2} s^{-1} or 1000 µmol photon m^{-2} s^{-1} for shade- and sun-acclimated plants, respectively. Additional leaves of sun-acclimated plants were also subjected to 100 µmol photon m^{-2} s^{-1} and shade-acclimated plants to 1000 µmol photon m^{-2} s^{-1}. Once a new steady-state gas exchange was reached, light was turned off and leaf gas exchange logged until a new steady-state condition.

Stomatal opening time (t_{90} opening) following a step increase in PPFD was defined as the time to reach 90% the difference between the initial and maximum values of g_s, while stomatal closure time (t_{90} closing) following a step decrease in PPFD was defined as the time to reach 90% the difference between the initial and minimum values of g_s. We decided to define the speed of stomatal opening and closing in terms of durations, given that they reflect the behavior of individual stomata and are independent of the magnitude of maximum gas exchange (Deans et al., 2018), which strongly varies between sun- and shade-acclimated leaves.

Experimental design and statistical analysis

All measurements were performed using four individuals per species and light condition (n = 4). Data were tested for normality through one-sample Kolmogorov–Smirnov test and, when

normality assumptions were not found, non-parametric tests were performed. Parameters from sun- and shade-acclimated plants were tested using unpaired Student *t*-tests ($P < 0.05$), while parameters from sun-acclimated plants at high and low PPFD and shade-acclimated plants at low PPFD were tested by Kruskal-Wallis followed by multiple comparison testing using Mann-Whitney U-tests ($P < 0.05$). Statistical analyses were performed and plots were constructed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA).

Results

Shading resulted in larger unitary leaf area in the two angiosperms, *E. urophylla* and *C. chinense*, but not in the conifer *P. macrophyllus* as well as in larger specific leaf area regardless of species (Figure 1). The *SD* of all three species decreased with shading, however, SS remained similar between sun- and shade-acclimated plants for *P. macrophyllus* and *C. chinense.* In *E. urophylla*, shading decreased the *SS* by over 25%.

The $t_{90opening}$ obtained when shade-acclimated plants were exposed to a step change in light from dark to low PPFD was similar to that observed when sun-acclimated plants were exposed to a step change in light from dark to both low and high PPFD, for all three species (Figure 2A-D). Similar results were observed for the $t_{90closing}$ of the conifer *P. macrophyllus* and the angiosperm *E. urophylla* (Figure 2E-H). For *C. chinense*, however, the $t_{90closing}$ of shade-acclimated plants was slower than that of sun-acclimated ones when both groups of plants were exposed to a step decrease in light from low irradiance to dark (Figure 2E,H). For the three species, $t_{90closing}$ was faster than $t_{90opening}$.

Overall, under growth PPFD, shade-acclimated plants experienced considerably lower leaf gas exchange rates (A and g_s) over the sun-acclimated ones (Figure 3A-B). When measuring leaf gas exchange under laboratory conditions, A and g_s of all shade-acclimated species under low PPFD were similar to that of sun-acclimated plants under the same low PPFD but lower than that of sun-acclimated individuals illuminated with high PPFD (Figure 3C-D). Alike leaf gas exchange, for both angiosperms, the K_{leaf} of shade-acclimated plants under low PPFD were comparable with that of sun-acclimated plants under low PPFD but lower than that of sun-acclimated plants under high PPFD (Figure 3E). For *P. macrophyllus*, K_{leaf} was similar between shade-acclimated plants under low PPFD and sun-acclimated plants under high PPFD, and irresponsive to dynamic chances in PPFD in sun-acclimated plants (Figure 3E). A linear and positive correlation ($r^2 = 0.95$, $P < 0.001$)

was observed between K_{leaf} and g_s, when all data obtained in the laboratory were analyzed (Figure 3F).

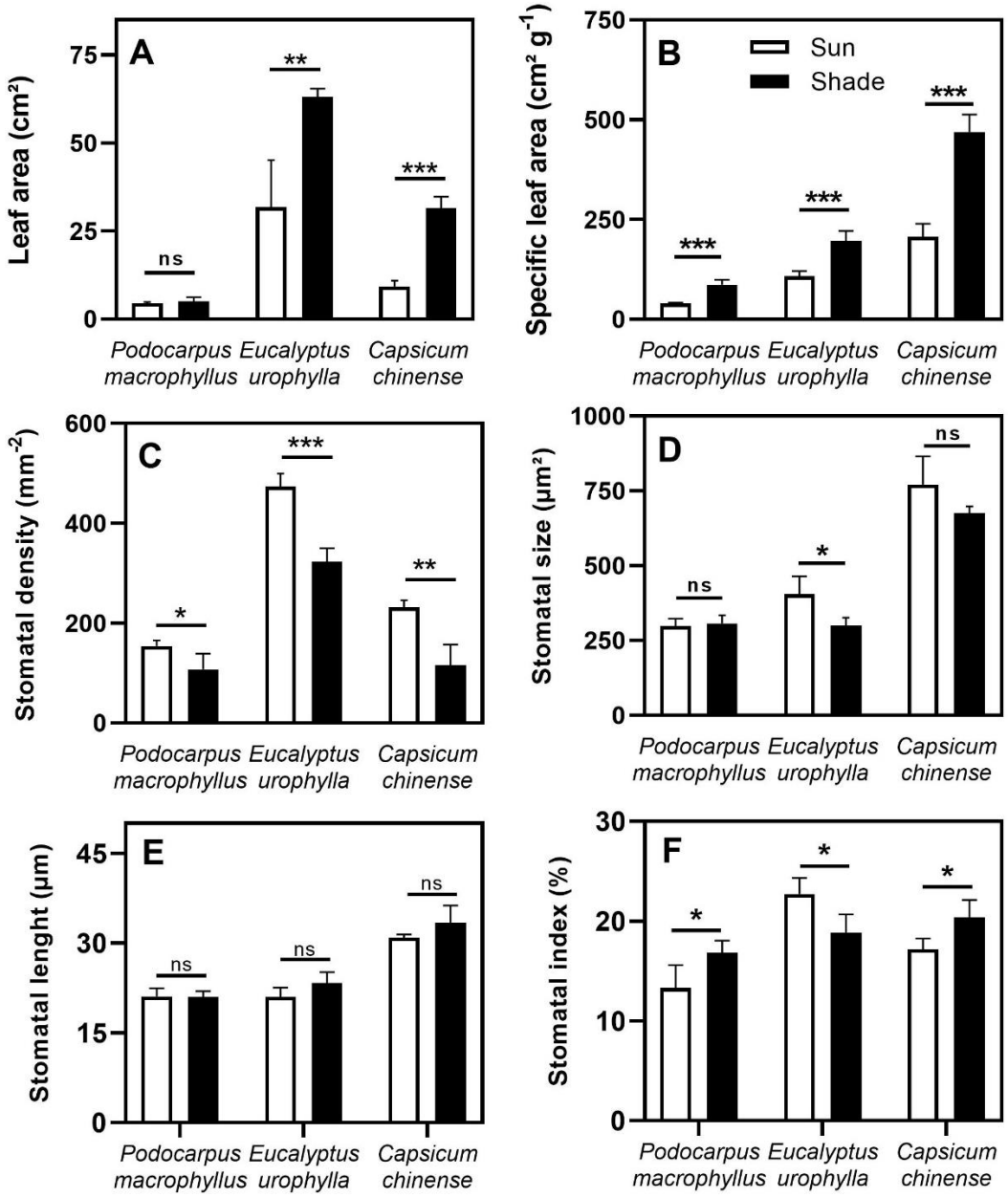

Figure 1. (A) Leaf area, (B) specific leaf area, (C) stomatal density, (D) stomatal size, (E) stomatal length and (F) stomatal index of three woody species cultivated in either sun or shade. Data are means ± SD (n = 4). Results of unpaired Student t-tests are indicated above columns (***, P < 0.001; **, P < 0.01; *, P < 0.05; ns, P > 0.05).

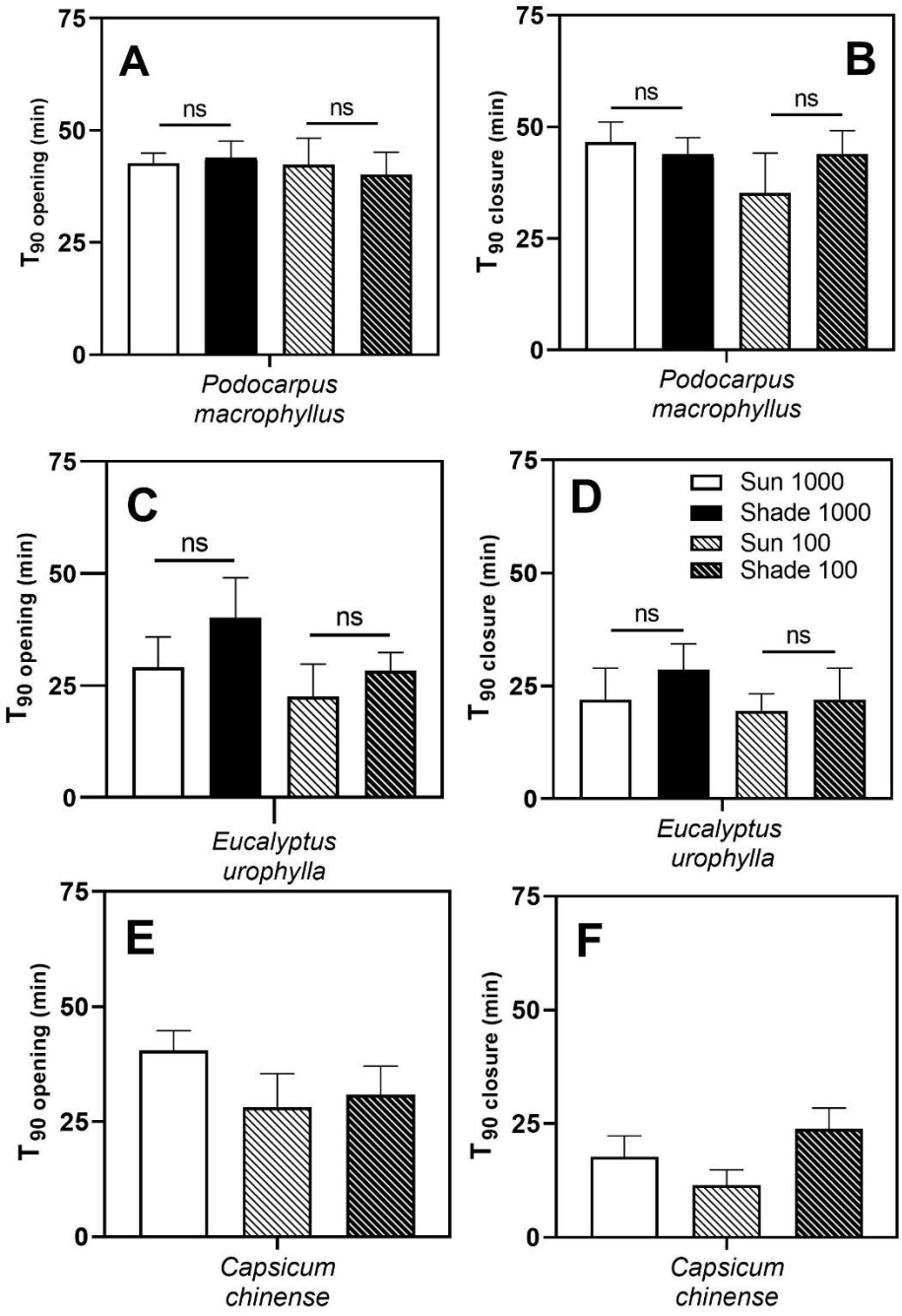

Figure 2. (A-C-E) Stomatal opening (t_{90} opening) and (B-D-F) stomatal closing (t_{90} closing) response time of three woody species cultivated at either sun or shade conditions. The responses of sun- and shade-acclimated plants were assessed at both low (100 µmol photons m^{-2} s^{-1}) and high (1000 µmol photons m^{-2} s^{-1}) photosynthetic photon flux density (PPFD). Data are means ± SD (n = 4). Results of unpaired Student t-tests are indicated above columns (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$).

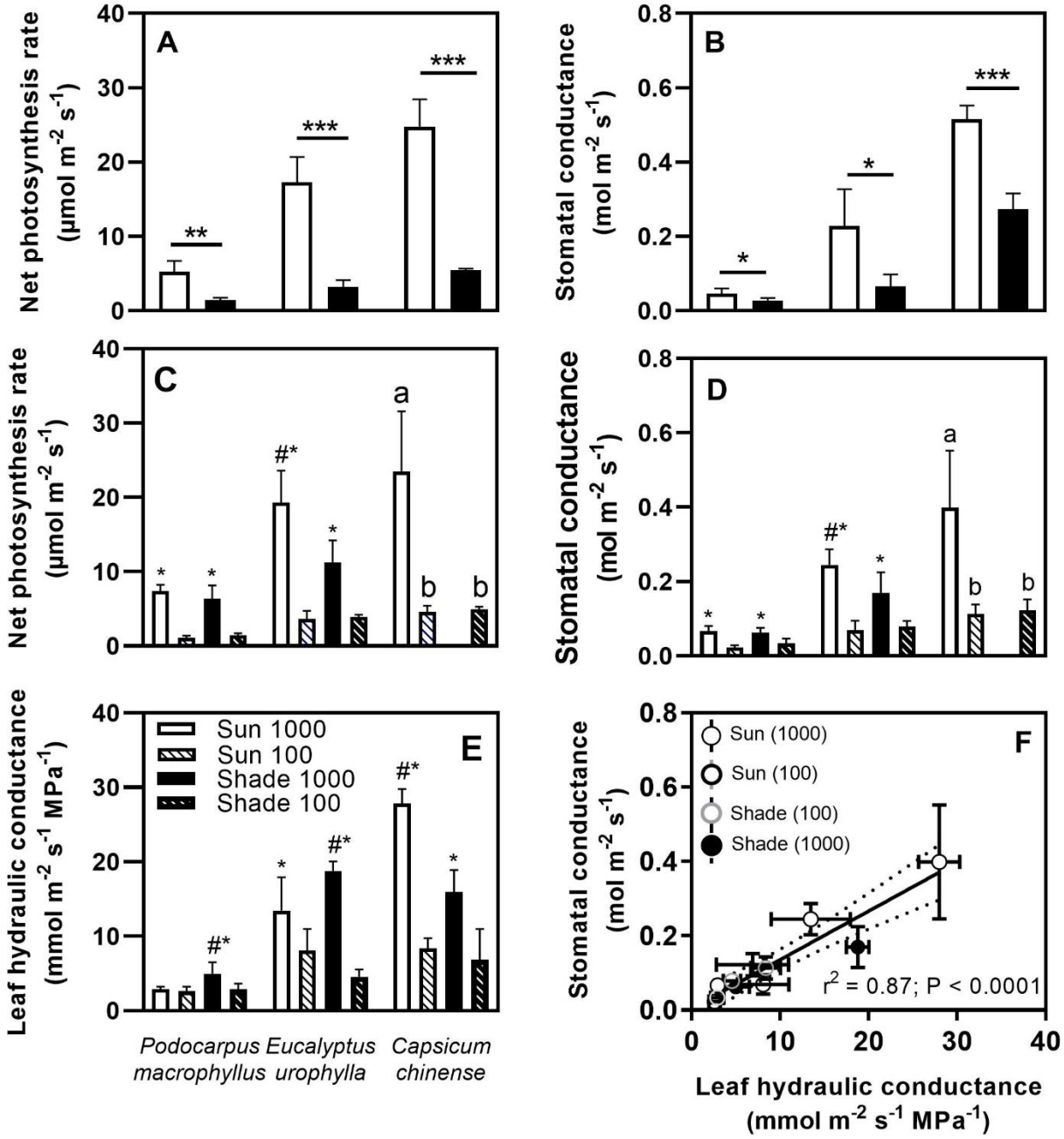

Figure 3. (A) Net photosynthesis rate and (B) stomatal conductance under natural growth conditions as well as (C-D) under controlled conditions at different PFFD using the LI-COR (data from curves depicted in Figure 2), (E) leaf hydraulic conductance, and (F) the relationship between leaf hydraulic conductance and stomatal conductance of three woody species cultivated at either sun or shade conditions. The responses of sun- and shade-acclimated plants were assessed at both low (100 μmol photons m^{-2} s^{-1}) and high (1000 μmol photons m^{-2} s^{-1}) photosynthetic photon flux

density (PPFD) . Data are means ± SD (n = 4). Asterisks above columns in (A-B) indicate differences according to unpaired Student t-tests (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$). Letters above columns of each species in (C-E) indicate groupings according to multiple comparison testing using Mann–Whitney U-tests ($P < 0.05$). Asterisks (*), when shown, indicate differences between the same growth PPFD in different light irradiance regimes; hashtags (#), when shown, indicate differences between the same light irradiance regimes at different growth PPFD. ($P \leq 0.05$, F test, n = 4 ± SE).

Discussion

Interspecific declines in SD with shading are independent of increases in SS

Like several other woody angiosperms (Carins Murphy *et al.*, 2012, 2016; Martins *et al.*, 2014), we observed an increase in area in leaves that developed under shade for the two angiosperms. Such foliar area increase is likely a result from increases in epidermal cell size which is consistent with the considerable decreases in *SD* observed in these angiosperms; such decreases are expected to occur due to a passive dilution mechanism as epidermal cells get larger (Carins Murphy *et al.*, 2012, 2016). Conversely, leaves of the conifer *P. macrophyllus* acclimated to sun and shade exhibited similar areas and, yet, its shade-acclimated leaves exhibited lower *SD* than their sun-acclimated counterparts. Light inducing plasticity in morpho-anatomical traits is less pronounced in moderate and high-tolerant shade species (Gratani, 2014), being these traits highly adjustable to shade conditions after long-term shade exposure. In this context, *P. macrophyllus*, which is a shade-tolerant species, does not exhibit significant plasticity in some attributes and those that manifest some degree of plasticity, this degree is lower compared to the values we observed for *E. urophylla* and *C. chinense*. In this case, the lower *SD* observed in shade-acclimated *P.*

macrophyllus plants cannot be explained by the passive dilution of stomata due to increases in epidermal cells. Instead, we hypothesize that a lower stomatal initiation took place in *P. macrophyllus* under shade (Hronková *et al.*, 2015; Lee *et al.*, 2017; Wei *et al.*, 2020), resulting in the lower *SD* of these plants over the sun-acclimated ones.

Unlike the ubiquitous decrease in *SD* with shading herein observed, *SS* exhibited a much more limited plasticity in response to growth PPFD. Out of the three species, only *E. urophylla* exhibited significant changes in *SS* with shading (a decrease in *SS* by c. 26%), while *SS* did not vary significantly for the other two species between plants acclimated to high and low PPFD. In addition, no species exhibit a significant plasticity in guard cell length in response to growth PPFD (Figure 1). The insensitivity of *SS* to changes in PPFD have been previously documented for woody and herbaceous species (Carins Murphy *et al.*, 2012, 2016; Martins *et al.*, 2014; Kardiman & Ræbild, 2018), while smaller stomata have also been observed in response to shading for herbaceous ferns and angiosperms (Carins Murphy *et al.*, 2016; Murphy *et al.*, 2017). Together with results from the aforementioned studies, our findings demonstrate that the plasticity in *SS* in response to light is species-specific, yet the mechanisms underlying either the maintenance or reductions in *SS* remain elusive.

It is noteworthy that declines in *SD* with shading in all three species are either uncoupled from changes in *SS* or coupled with decreases in *SS* (Figure 1; Supplementary Figure 4A), adding to a growing body of evidence that the interspecific alterations of both traits in response to light are independent (Carins Murphy *et al.*, 2012, 2016; Martins *et al.*, 2014; Murphy *et al.*, 2017; Kardiman & Ræbild, 2018). Particularly for the case of *E. urophylla*, the lack of positive correlation between *SD* and *SS* contrasts with that found in *E. globulus* across environmental gradients (Franks *et al.*, 2009). This reinforces the idea that changes in the size of epidermal cells

happen independently of changes in guard cells, possibly because the final stomatal size takes place well before the final expansion of the leaf (Schoch *et al*., 1980; Zwieniecki *et al.,* 2004; Carins Murphy *et al.*, 2012).

Times for stomatal opening and closure are essentially insensitive to growth light

Stomatal responsivity to changes in light supply have been characterized at inter- and intraspecific levels (Vico *et al*., 2011; McAusland *et al*., 2016; Faralli *et al*., 2019; Eyland *et al*., 2021). In addition to light responsiveness, these changes also depend on alterations in soil water potentials (Meinzer *et al.,* 2017; Faralli *et al*., 2019) and environmental concentrations of CO_2 (Zhang *et al*., 2018). Moreover, changes in voltage-dependent potassium influx channels also had their importance elucidated (Papanatsiou *et al*., 2019). These changes ultimately have profound impacts on water use efficiency, carbon assimilation efficiency, and maintenance of growth rates, and deeper insights about these topics were discussed in the aforementioned literature. It is noteworthy that despite stomatal responses present a profound sensitivity to a myriad of environmental stimuli, this mechanism did not vary in our study due to changes in growth PPFD or when sun-acclimated plants had their stomatal speed measured at low PPFD. Dealing with plants in seasonally or permanently shaded conditions (Liu *et al.,* 2016; Gommers *et al.,* 2013; Valladares & Niinemets; 2008) the maintenance of the stomatal speed tends to trigger an unbalance in the valve stomatal activity, which in turn maintains the CO_2 uptake rates at the expense of accentuated loss of water vapor. This continuum carbon uptake probably is not readily available for photosynthesis given that, under these conditions, reductions on photosynthetic activity are observed due to restrictions in light supply (Mathur *et al.,* 2018; Valladares & Niinemets; 2008).

Previous studies have discussed the implications related to changes in stomatal speed (Kardiman & Ræbild, 2018; Eyland *et al.,* 2021). In seasonally shaded environments, the unaltered stomatal speed to growth PPFD changes could allow immediate photosynthetic rates when rare sun flacks appear on the leaves, thus reducing the need for costly stomatal adjustments. On the other hand, in permanently shaded (humid) environments, where drought stress risk is smaller, it could guarantee high photosynthetic rates. In this context, we believe that changes in stomatal speed is linked to the behavioral ecology of plant species, seeing that in our study, organisms with different needs exhibited similar stomatal speed with a step increase in irradiance inducing stomatal opening and in face of changes in PPFD growth, even though they present contrast maximum values of stomatal opening. This seems plausible because Kardiman & Ræbild (2018) observed a higher response speed during the opening in sun-acclimated plants of *Aquilaria malaccensis*, while for *Macaraga triloba* the speed was lower, but for *Eusideroxylon zwageri* and *Eusideroxylon zwageri* no changes were observed, these results partly align and partly contradict those observed here, showing that the time required for changes in stomatal speed seems to be a trait under a strong genetic control and do not exhibit feisty changes to growth PPFD.

Faster stomatal kinetics have been associated with greater *SD* and reduced *SS* due to their greater membrane surface area to volume ratio (Hetherington & Woodward, 2003; Franks & Beerling, 2009; Drake *et al.*, 2013; Kardiman & Ræbild, 2018), which facilitates the exchange of water and solutes between stomata and the cells around them, necessary for stomatal opening (Lawson & Blatt 2014). Moreover, some features correlated with a faster speed (e.g. K_{leaf} and *SD*) (Bertolino *et al*., 2019; Kardiman & Ræbild 2018) displayed plasticity to growth PPFD in this study. On the other hand, these changes were not necessarily translated into a faster stomatal opening speed. Despite high values of K_{leaf} and *SD* are important for optimized stomatal speed,

other factors assume greater importance in this process (Eyland *et al*., 2021). We suggest that other biochemical, structural, and molecular components of guard cells and vacuolar ionic dynamic transport play a major role in stomatal speed (Eyland *et al*., 2021; Lawson & Matthews, 2020; Medeiros *et al*., 2019), characteristics that are unaltered in face to changes to growth PPFD, albeit the exact mechanisms underlying this process are not immediately evident. In addition, observing plants acclimated to high PPFD having their stomatal speed measured under low PPFD, we have observed one more indication that some properties that govern stomatal movements seem to be insensitive to light. Despite some studies have devoted efforts to elucidate how vacuolar channels and transporters command stomatal movements (Eisenach & Angeli, 2017), and how changes in the activity of these channels impact the opening and closing of stomata (Yang *et al*., 2020; Medeiros *et al*., 2016), doubts remain regarding the response of these components under low PPFD in comparison with exposure to high PPFD.

Conclusions

As a whole, here we demonstrate that the light condition at which woody species are grown result in a ubiquitous variation in morpho-anatomical and hydraulic properties in leaves. Our findings show that there is a differential plasticity between species. The time for stomatal responses to light is essentially insensitive to the changes in leaf structure associated with the light condition during growth. Our results suggest that both stomatal size and speed exhibit limited plasticity in response to light across woody species and are likely under strong genetic control.

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References

Baroli I, Price GD, Badger MR, Von Caemmerer S. **2008**. The contribution of photosynthesis to the red light response of stomatal conductance. *Plant Physiology* **146**: 737–747.

Bertolino LT, Caine RS, Gray JE. 2019. Impact of stomatal density and morphology on water-use efficiency in a changing world. Frontiers in Plant Science. **10**: 225.

Brodribb TJ, Holbrook NM. 2006. Declining hydraulic efficiency as transpiring leaves desiccate: two types of response. *Plant, Cell and Environment* **29**: 2205-2215.

Brodribb TJ, Feild TS, Jordan GJ. **2007**. Leaf maximum photosynthetic rate and venation are linked by hydraulics. *Plant Physiology* **144**: 1890–1898.

Brodribb TJ, McAdam SAM. **2017**. Evolution of the stomatal regulation of plant water content. *Plant Physiology* **174**: 639–649.

Brodribb TJ, Sussmilch F, McAdam SAM. **2020**. From reproduction to production, stomata are the master regulators. *Plant Journal* **101**: 756–767.

Carins Murphy MR, Jordan GJ, Brodribb TJ. **2012**. Differential leaf expansion can enable hydraulic acclimation to sun and shade. *Plant Cell and Environment* **35**: 1407–1418.

Carins Murphy MR, Jordan GJ, Brodribb TJ. **2016**. Cell expansion not cell differentiation predominantly co-ordinates veins and stomata within and among herbs and woody angiosperms

grown under sun and shade. *Annals of Botany* **118**: 1127–1138.

Chen ZH, Eisenach C, Xu XQ, Hills A, Blatt MR. **2012**. Protocol: optimised electrophyiological analysis of intact guard cells from Arabidopsis. *Plant Methods* **8**: 1-11.

Deans RM, Brodribb TJ, Busch FA, Farquhar GD. **2018**. Plant water-use strategy mediates stomatal effects on the light induction of photosynthesis. *New Phytologist* **222**: 382–395.

Drake PL, Froend RH, Franks PJ. **2013**. Smaller, faster stomata: Scaling of stomatal size, rate of response, and stomatal conductance. *Journal of Experimental Botany* **64**: 495–505.

Eisenach C, Angeli A. 2017. Ion transport at the vacuole during stomatal movements. *Plant Physiology* **174**: 520 - 530.

Eisenach C, Chen ZH, Grefen C, Blatt MR. **2012**. The trafficking protein SYP121 of Arabidopsis connects programmed stomatal closure and K+ channel activity with vegetative growth. *Plant Journal* **69**: 241–251.

Eisenach C, Papanatsiou M, Hillert EK, Blatt MR. **2014**. Clustering of the K+ channel GORK of Arabidopsis parallels its gating by extracellular K+. *Plant Journal* **78**: 203–214.

Elliott-Kingston C, Haworth M, Yearsley JM, Batke SP, Lawson T, McElwain JC. **2016**. Does size matter? Atmospheric CO2 may be a stronger driver of stomatal closing rate than stomatal size in taxa that diversified under low CO2. *Frontiers in Plant Science* **7**: 1 - 12.

Eyland D, Wesemael JV, Lawson T, Carpentier S. 2021. The impact of slow stomatal kinetics on photosynthesis and water use efficiency under fluctuating light. *Plant Physiology* **186**: 998–1012.

Faralli M, Cockram J, Ober E, Wall S, Galle A, Rie JV, Raines C, Lawson T. 2019. Genotypic, developmental and environmental effects on the rapidity of gs in wheat: impacts on carbon gain and water-use efficiency. *Frontiers in Plant Science* **10**: 1-13.

Franks PJ, Beerling DJ. **2009**. Maximum leaf conductance driven by CO2 effects on stomatal size and density over geologic time. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 10343–10347.

Franks PJ, Drake PL, Beerling DJ. **2009**. Plasticity in maximum stomatal conductance constrained by negative correlation between stomatal size and density: An analysis using *Eucalyptus globulus*. *Plant, Cell and Environment* **32**: 1737–1748.

Franks PJ, Farquhar GD. **2007**. The mechanical diversity of stomata and its significance in gas-exchange control. *Plant Physiology* **143**: 78–87.

Gerardin T, Douthe C, Flexas J, Brendel O. **2018**. Shade and drought growth conditions strongly impact dynamic responses of stomata to variations in irradiance in *Nicotiana tabacum*. *Environmental and Experimental Botany* **153**: 188–197.

Gommers CM, Visser EJ, St Onge KR, Voesenek LA, Pierik R. 2013. Shade tolerance: when growing tall is not an option. *Trends in Plant Science*, **18**: 65-71.

Gratani L. 2014. Plant phenotypic plasticity in response to environmental actors. *Advances in Botany*. **2014**: 1-17.

Hetherington AM, Woodward FI. **2003**. The role of stomata in sensing and driving environmental change. *Nature* **424**: 901–908.

Hronková M, Wiesnerová D, Šimková M, Skůpa P, Dewitte W, Vráblová M, Zažímalová E,

Šantrůček J. **2015**. Light-induced STOMAGEN-mediated stomatal development in Arabidopsis leaves. *Journal of Experimental Botany* **66**: 4621–4630.

Inoue SI, Kinoshita T. **2017**. Blue light regulation of stomatal opening and the plasma membrane H+-ATPase. *Plant Physiology* **174**: 531–538.

Kardiman R, Ræbild A. **2018**. Relationship between stomatal density, size and speed of opening in Sumatran rainforest species. *Tree Physiology* **38**: 696–705.

Knapp AK, Smith WK. **1987**. Stomatal and photosynthetic responses during sun/shade transitions in subalpine plants: influence on water use efficiency. *Oecologia* **74**: 62–67.

Korson L, Drost-Hansen W, Millero FJ. 1969. Viscosity of water at various temperatures. *Journal of Physical Chemistry* **73**: 34–39.

Lawson T, Blatt MR. **2014**. Stomatal size, speed, and responsiveness impact on photosynthesis and water use efficiency. *Plant Physiology* **164**: 1556–1570.

Lawson T, Matthews J. 2020. Guard cell metabolism and stomatal function. *Annual Review of Plant Biology* **71**: 273 - 302.

Lee JH, Jung JH, Park CM. **2017**. Light inhibits COP1-mediated degradation of ICE transcription factors to induce stomatal development in arabidopsis. *The Plant Cell* **29**: 2817–2830.

Liu Y, Dawson W, Prati D, Haeuser E, Feng Y, van Kleunen M. 2016. Does greater specific leaf area plasticity help plants to maintain a high performance when shaded? *Annals of Botany*, **118**: 1329-1336.

Martins SCV, Galmés J, Cavatte PC, Pereira LF, Ventrella MC, DaMatta FM. **2014**. Understanding the low photosynthetic rates of sun and shade coffee leaves: Bridging the gap on

the relative roles of hydraulic, diffusive and biochemical constraints to photosynthesis. *PLoS ONE* **9**: 1–10.

Mathur S, Jain L, Jajoo A. 2018. Photosynthetic efficiency in sun and shade plants. *Photosynthetica* **56**: 354-365.

Matthews JSA, Lawson T. **2019**. Climate Change and Stomatal Physiology. *Annual Plant Reviews* **2**: 1–39.

Matthews JSA, Vialet-Chabrand S, Lawson T. **2018**. Acclimation to fluctuating light impacts the rapidity of response and diurnal rhythm of stomatal conductance. *Plant Physiology* **176**: 1939–1951.

McAusland L, VialetChabrand S, Davey P, Baker NR, Brendel O, Lawson T. **2016**. Effects of kinetics of light-induced stomatal responses on photosynthesis and water-use efficiency. *New Phytologist* **211**: 1209-1220.

Medeiros DB, Martins SCV, Cavalcanti JHF, Daloso DM, Martinoia E, Nunes-Nesi A, DaMatta FM, Fernie AR, Araújo WL. 2016. Enhanced photosynthesis and growth in atquac1 knockout mutants are due to altered Organic acid accumulation and an increase in both stomatal and mesophyll conductance. *Plant Physiology* **170**: 86–101.

Medeiros DB, Luz LM, Oliveira HO, Araújo WL, Daloso DM, Fernie AR. 2019. Metabolomics for understanding stomatal movements. *Theoretical and Experimental Plant Physiology* **31**: 91–102.

Meinzer FC, Smith DD, Woodruff DR, Marias DE, McCulloh KA, Howard AR, Magedman AL. 2017. Stomatal kinetics and photosynthetic gas exchange along a continuum of isohydric to anisohydric regulation of plant water status. *Plant, Cell and Environment* **40**: 1618-1628**.**

Murphy MRC, Jordan GJ, Brodribb TJ. **2017**. Ferns are less dependent on passive dilution by cell expansion to coordinate leaf vein and stomatal spacing than angiosperms. *PLoS ONE* **12**: e0185648.

Papanatsiou M, Petersen J, Henderson L, Wang Y, Christie JM, Blatt MR. 2019. Optogenetic manipulation of stomatal kinetics improves carbon assimilation, water use, and growth. *Science* **363**:1456–1459.

Rodríguez-López NF, Martins SCV, Cavatte PC, Silva PEM, Morais LE, Pereira LF, Reis J V., Ávila RT, Godoy AG, Lavinski AO, *et al.* 2014. Morphological and physiological acclimations of coffee seedlings to growth over a range of fixed or changing light supplies. *Environmental and Experimental Botany* **102**: 1–10.

Sack L, Melcher PJ, Zwieniecki MA, Holbrook NM. 2002. The hydraulic conductance of the angiosperm leaf lamina: a comparison of three measurement methods. *Journal of Experimental Botany*, **53**: 2177-2184.

Schoch PG, Zinsou C, Sibi M. 1980. Dependence of the stomatal index on environmental factors during stomatal differentiation in leaves of *Vigna sinensis* L. *Journal of Experimental Botany* **31**: 1211–1216.

Scoffoni C, Pou A, Aasamaa K, Sack L. **2008**. The rapid light response of leaf hydraulic conductance: new evidence from two experimental methods. *Plant, Cell and Environment* **31**: 1803–1812.

Shimazaki KI, Doi M, Assmann SM, Kinoshita T. **2007**. Light regulation of stomatal movement. *Annual Review of Plant Biology* **58**: 219–247.

Strittmatter CGD. 1973. Nueva técnica de diafanización. *Boletín de la Sociedad Argentina de Botánica* **15**: 126–129.

Valladares F, Niinemets Ü. 2008. Shade tolerance, a key plant feature of complex nature and consequences. *Annual Review of Ecology, Evolution, and Systematics* **39**: 237–257.

Vico G, Manzoni S, Palmroth S, Katul G. **2011**. Effects of stomatal delays on the economics of leaf gas exchange under intermittent light regimes. *New Phytologist* **192**: 640–652.

Wei H, Kong D, Yang J, Wang H. **2020**. Light regulation of stomatal development and patterning: shifting the paradigm from *Arabidopsis* to grasses. *Plant Communications* **1**: 100030.

Woods DB, Turner NC. **1971**. Stomatal response to changing light by four tree species of varying shade tolerance. *New Phytologist* **70**: 77–84.

Yang J, Li C, Kong D, Guo F, Wei H. 2020. Light-mediated signaling and metabolic changes coordinate stomatal opening and closure. *Frontiers in Plant Science* **11**: 601478.

Zhang J, De-oliveira-Ceciliato P, Takahashi Y, Schulze S, Dubeaux G, Hauser F, Azoulay-Shemer T, Toldsepp K, Kollist H, Rappel WJ, Schroeder JI. 2018. Insights into the Molecular Mechanisms of CO_2-Mediated Regulation of Stomatal Movements. *Current Biology* **23**: 1356 - 1363.

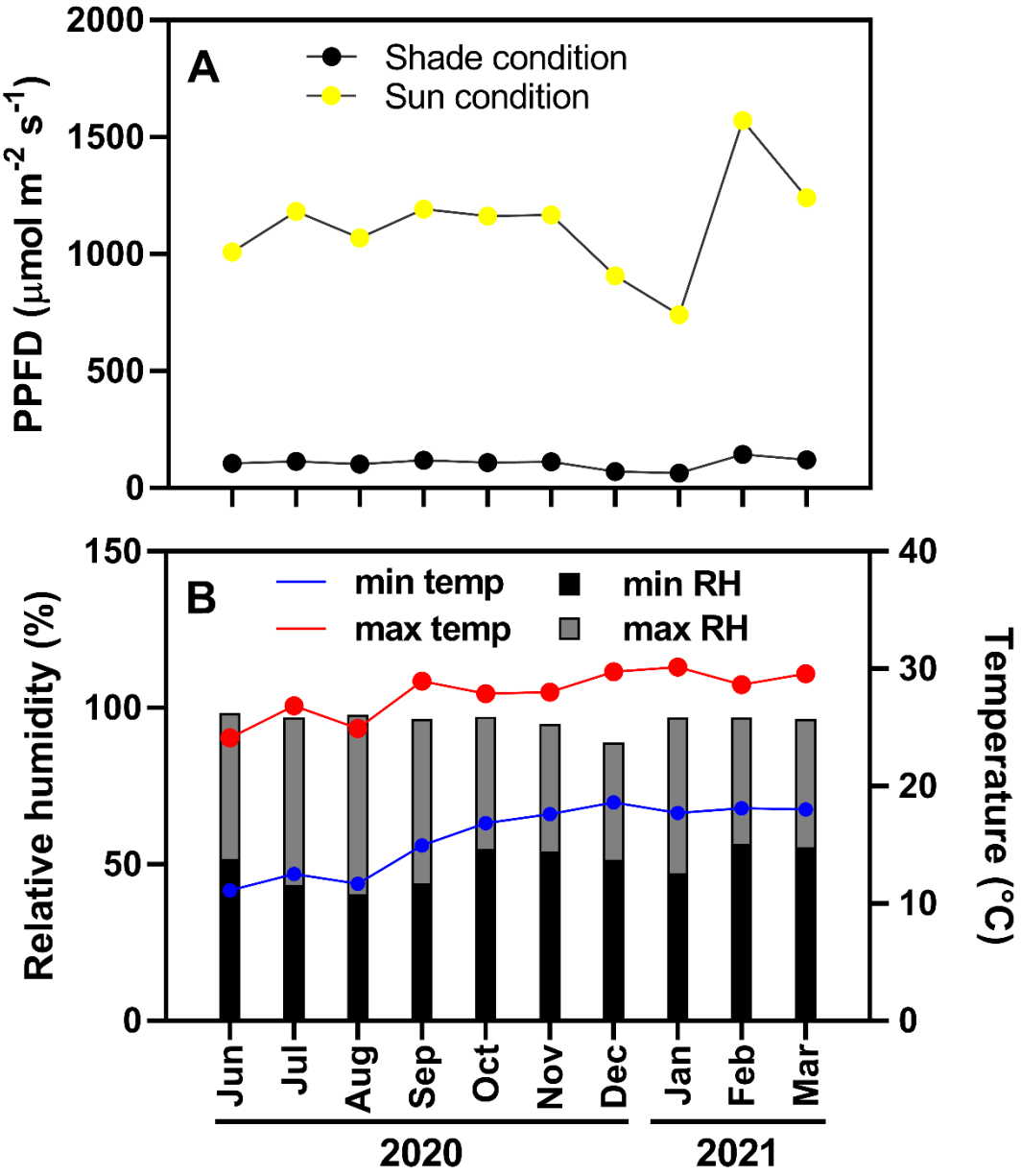
Zwieniecki MA, Boyce CK, Holbrook NM. 2004. Hydraulic limitations imposed by crown placement determine final size and shape of *Quercus rubra* L. leaves. *Plant, Cell and Environment* **27**: 357–365

Supplementary Information

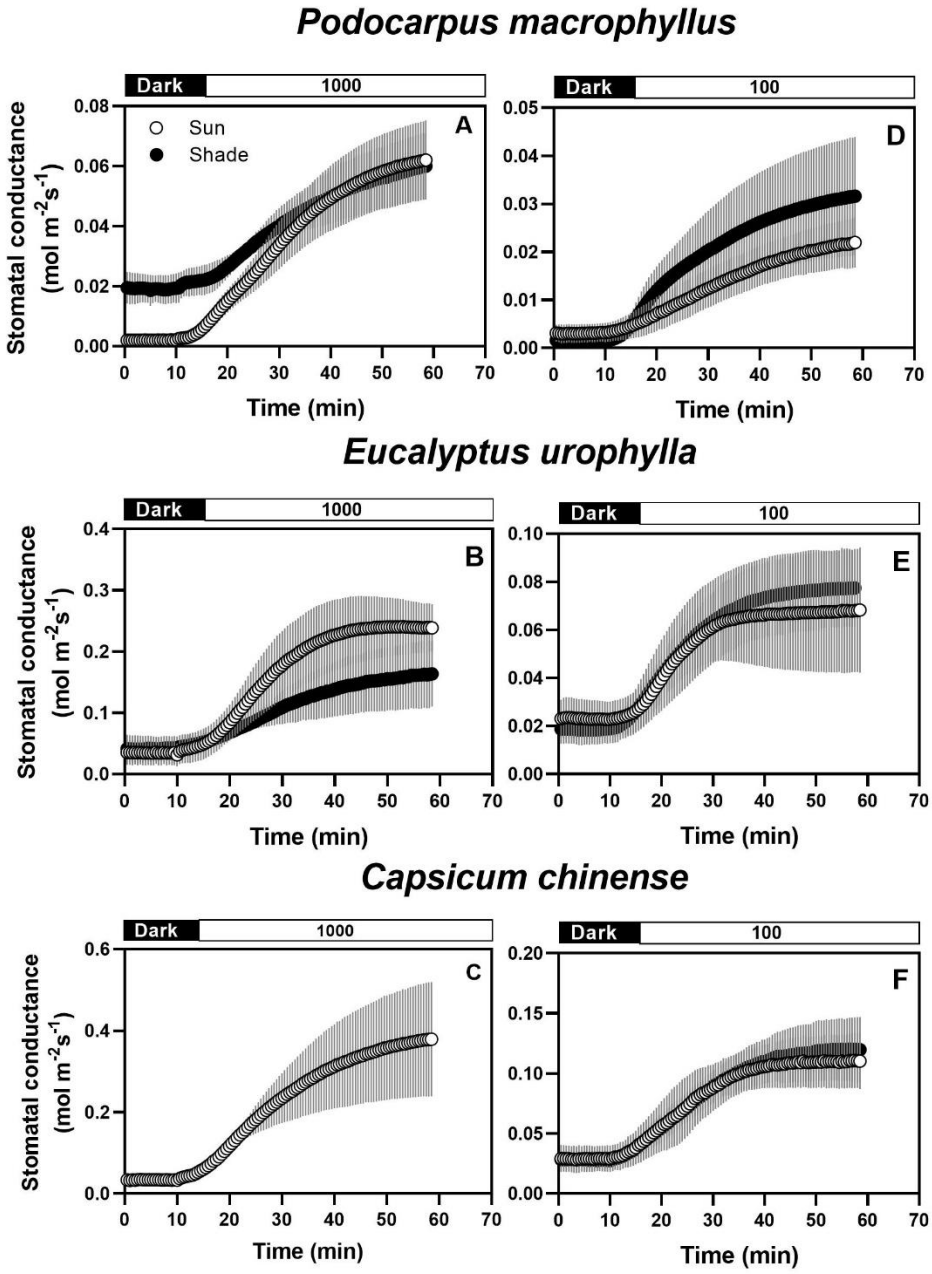
Supplementary Table 1. Mean predawn and midday water potentials (MPa) of three woody species cultivated in either sun or shade. Data are means of four individuals (n = 4) obtained at three different sunny days ± SD.

Traits	Predawn water potential		Midday water potential	
	Sun	**Shade**	**Sun**	**Shade**
Podocarpus macrophyllus	- 0.10 ± 0.007	- 0.09 ± 0.004*	- 0.59 ± 0.09	- 0.55 ± 0.04**
Eucalyptus urophylla	- 0.09 ± 0.001	- 0.08 ± 0.004*	- 1.15 ± 0.08	- 0.74 ± 0.06**
Capsicum chinense	- 0.07 ± 0.003	- 0.07 ± 0.004^{ns}	- 0.56 ± 0.03	- 0.45 ± 0.04*

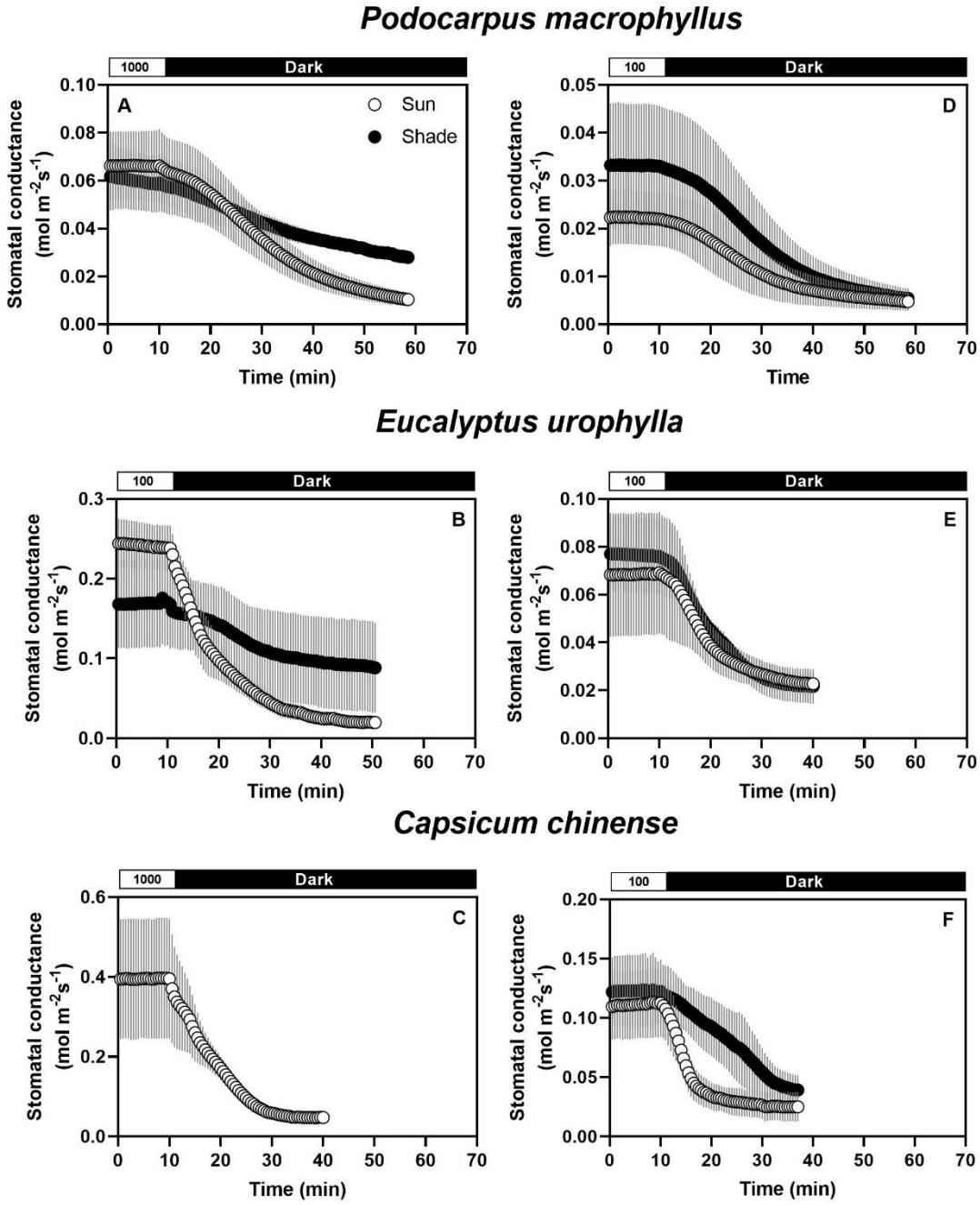
Asterisks indicate differences between sun- and shade-acclimated plants during either predawn or midday according to unpaired Student t-tests (**, $P < 0.01$; *, $P < 0.05$; ns, $P > 0.05$).



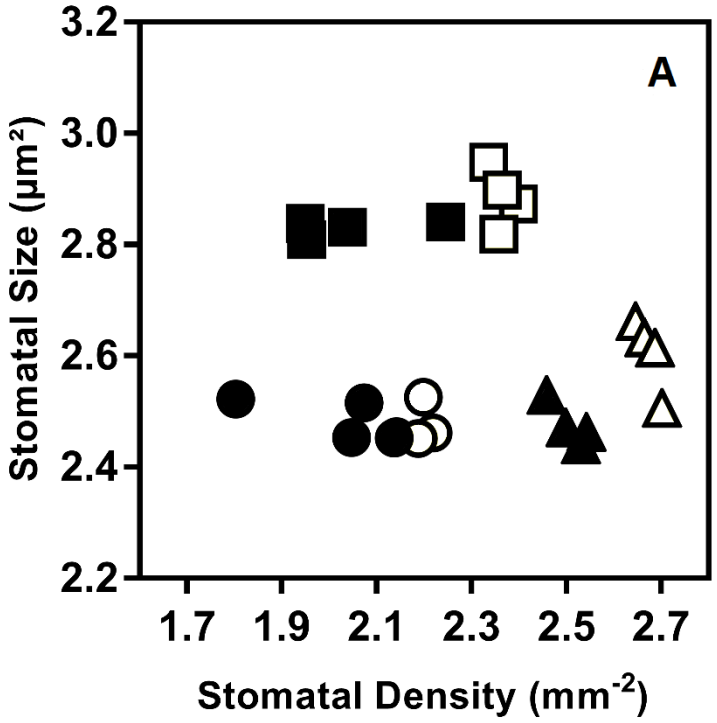
Supplementary Figure 1. (A) Mean (n = 4 weeks) maximum photosynthetic photon flux density (PPFD) of sun and shade conditions measured between 11:00 and 13:00 h (solar time) and (B) mean minimum and maximum relative humidity and temperature of the experimental site from June 2020 throughout March 2021.



Supplementary Figure 2. Curves showing stomatal aperture from dark to light of three woody species cultivated at either sun or shade conditions. The responses of sun- and shade-acclimated plants were assessed at both low (100 μmol photons m^{-2} s^{-1}) and high (1000 μmol photons m^{-2} s^{-1}) photosynthetic photon flux density (PPFD). Data are means ± ± SD (n = 4).



Supplementary Figure 3. Curves showing stomatal closure curves showing stomatal closure light to dark of three woody species cultivated at either sun or shade conditions. The responses of sun and shade-acclimated plants were assessed at m^{-2} s^{-1} (100 µmol photons m^{-2} s^{-1}) and high photosynthetic photon flux density (PPFD) (1000 µmol photons m^{-2} s^{-1}). Data are means ± ± SD (n = 4).



Supplementary Figure 4. (A) Relationship between stomatal density and stomatal size of three woody species cultivated in either sun or shade. Points are data from individual leaves (white for sun and black for shade) of *P. macrophyllus* (circle), *E. urophylla* (triangles), and *C. chinense* (square).