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Woody species grown under sun and shade present similar stomatal speed

Rafael S. Freitas · Leonardo A. Oliveira · Scott A. M. McAdam · Tracy Lawson · Fábio M. DaMatta · Amanda A. Cardoso

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Abstract 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 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. We demonstrate that intense shading conditions promote high levels of plasticity in the woody species 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 these species, however, did not translate into alterations in the time to reach 90% of the maximum stomatal conductance (g_s) changes (t_{90}) when plants were exposed to dynamic changes in irradiance. In some cases, the growth light affected the maximum slope of g_s. This approach, however, was demonstrated not to be ideal for assessing stomatal speed in sun- and shade-acclimated plants as this method was largely dependent on maximum g_s. Our findings suggest that stomatal speed, as demonstrated by t_{90}, has low phenotypic plasticity and is most likely under a stronger genetic regulation than other leaf and stomatal anatomical traits.

Keywords Irradiance · Leaf gas exchange · Light acclimation · Shade leaves · Stomatal dimensions · Stomatal speed · Sun leaves

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R. S. Freitas · L. A. Oliveira · F. M. DaMatta
Departamento de Biologia Vegetal, Universidade Federal de Viçosa, Viçosa, Brazil

L. A. Oliveira · A. A. Cardoso (✉)
Department of Crop and Soil Sciences, North Carolina State University, Raleigh, NC 27695, USA
e-mail: aavilac@ncsu.edu

S. A. M. McAdam
Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47906, USA

T. Lawson
School of Life Sciences, University of Essex, Colchester CO4 4SQ, UK

1 Introduction

Stomata are tiny pores surrounded by a pair of specialized guard cells that actively regulate the majority of gaseous diffusion between the leaf interior and the atmosphere (Franks and 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 and McAdam 2017; Matthews and Lawson

2019). Amongst all environmental cues regulating stomatal aperture, light appears as the most pervasive one—a step increase in irradiance induces stomatal opening and a step decrease causes stomata to close in nearly all vascular land plants (Deans et al. 2018). 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 and Kinoshita 2017; Vialet-Chabrand et al. 2021).

In natural environments, the incident irradiance on leaves is not steady, constantly changing as clouds pass and higher leaves move shading the lower leaves and resulting in sun and shade flecks (Woods and 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 (Lawson et al. 2010; Vico et al. 2011; Lawson and Blatt 2014; McAusland et al. 2016; 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 (Lawson et al. 2010, 2012). 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 and Blatt 2014; Deans et al. 2018), eroding intrinsic water use efficiency (McAusland et al. 2016; Faralli et al. 2019). 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 and Blatt 2014; McAusland et al. 2016).

Across different plant species, a significant variation in the speed 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 and Ræbild 2018; Eyland et al. 2021), but the mechanisms controlling stomatal speed are far from being understood (Lawson and 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 and Woodward 2003; Franks and Beerling 2009; Drake et al. 2013; Kardiman and Ræbild 2018). This hypothesis has been drawn based on the assumption that transport activity of guard cells, 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 between 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 and Turner 1971; Deans et al. 2018; Kardiman and 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 under a canopy (Woods and Turner 1971; Knapp and Smith 1987). Despite several studies comparing the speed of stomatal responses to changing light across species over the past decades, studies assessing the plasticity of stomatal speed in response to environmental conditions are much more recent and far less common (Gerardin et al. 2018; Kardiman and Ræbild 2018; Matthews et al. 2018). Results from these studies range from limited plasticity of stomatal dynamics in response to light (Kardiman and Ræbild 2018) to varying degrees of plasticity in the speed and magnitude of stomatal responses (Gerardin et al. 2018; Kardiman and Ræbild 2018; Matthews et al. 2018), suggesting a species-specific mechanism, not yet fully understood.

The present study aimed at investigating the plasticity in stomatal speed during dynamic increases and decreases in light across woody species grown under contrasting light regimes (sun and shade)—a condition known to profoundly change leaf anatomy and function, including stomatal traits (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)—a shrub (Baruah and Lal 2020)] were selected. These species span a wide phylogenetic basis and they are able to grow under contrasting light irradiances. Specifically, we investigated whether growth light-induced changes in stomatal morphology would result in changes in stomatal speed in plants exposed

to dynamic changes in light. Among these morphological changes, we focused on stomatal density (*SD*), stomatal length (*SL*), *SS*, and leaf hydraulic conductance (K_{leaf}) (Scoffoni et al. 2008; Carins Murphy et al. 2016). Dynamic increases in K_{leaf} in response to increasing irradiance have been demonstrated for a number of species due to a higher expression and/or activation of aquaporins (Scoffoni et al. 2008). Stomatal speed was assessed using two methods: time to reach 90% of the maximum change in stomatal conductance (t_{90}) and maximal slope of stomatal conductance change (Sl_{max}). Our main hypothesis was that sun-acclimated leaves would achieve higher *SD* and K_{leaf} as well as lower *SS* and *SL*, thus leading to a faster stomatal movement in response to light.

2 Material and methods

2.1 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, MG (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 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 Fig. 1A). Plants grown at full sunlight received on average (± SE) a PPFD of 1120 ± 70 µmol photons m^{-2} s^{-1}, while plants under shade received a PPFD of c. 105 ± 10 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 Fig. 1B). All measurements described below were performed using four completely expanded leaves ($n = 4$) that were developed entirely in either full sunlight or shade.

2.2 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. Leaf dry mass was obtained by oven-drying leaves at 70 °C for 72 h. For anatomy purposes, leaves were fixed in FAA70 (formaldehyde, acetic acid, and 70% ethanol) for 48 h and then stored in 70% (v/v) 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 of 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*, stomatal index (*SI*), 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 the 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. The *SI* was calculated as the ratio of the stomatal number divided by total number of stomata and epidermal cells.

2.3 Stomatal dynamics

Prior to analyses of stomatal responses to light, plants were transferred to the laboratory and maintained in the dark overnight. During the next morning, leaves were enclosed in a conifer chamber (6400-22 L, 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 µL L^{-1}) and vapor pressure deficit (VPD) (c. 1.4 ± 0.1 kPa). Leaves were kept under these conditions until steady-state g_s and A were reached, after which gas exchange parameters were logged every 30 s. After 10 min of steady-state measurements under darkness, PPFD 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, the light was turned off and the leaf gas exchange was logged until a new steady-state condition.

We calculated stomatal responses using two methods: time to reach 90% of the maximum change in g_s (t_{90}) (Deans et al. 2018) and maximal slope of g_s change (SI_{max}) (McAdam and Brodribb 2015; Eyland et al. 2021) (Supplementary Fig. 2). The time to reach 90% of the maximum g_s increase ($t_{90opening}$) and the maximal slope of g_s increase ($SI_{opening}$) were assessed following a step increase in light from dark to either low (100 µmol photon m^{-2} s^{-1}) or high (1000 µmol photon m^{-2} s^{-1}) PPFD. The time to reach 90% of the maximum g_s decrease ($t_{90closure}$) and the maximal slope of g_s decrease ($SI_{closure}$) were assessed following a step decrease in light from either low (100 µmol photon m^{-2} s^{-1}) or high (1000 µmol photon m^{-2} s^{-1}) PPFD to dark.

2.4 Stomatal and leaf hydraulic conductance under low and high light

Leaf gas exchange was measured *in situ* from leaves from the uppermost parts of the canopy using the above-mentioned gas exchange analyzer. 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 µL L^{-1}) and vapor pressure deficit (VPD) (ranging from 0.5 to 1.5 kPa) were utilized.

The K_{leaf} was measured using the same leaves later utilized for leaf area and anatomy using the evaporative flux method and flowmeter (Sack et al. 2002; Brodribb and Holbrook 2006). Leaves were excised under water, immediately attached to a custom-built flowmeter, and placed in conditions to induce increased transpiration rates (i.e., under a light source and above a large cooler). The flowmeter measures the amount of water that is uptaken by leaves, which is a proxy for the amount of water that is lost through transpiration. 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 steady state in terms of the water absorption rate as measured though the flowmeter (less than 10% variation over 5 min), they were detached from the flowmeter, and had their water potential measured with a pressure chamber. The K_{leaf} was then calculated by dividing the water absorption rate at steady-state by the water potential. 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).

2.5 Experimental design and statistical analysis

The data were tested for normality using the one-sample Kolmogorov–Smirnov test. The parameters from sun- and shade-acclimated plants were tested using unpaired Student t-tests ($P < 0.05$), and when normality assumptions were not found, Mann–Whitney U test was performed. Statistical analyses were performed, and plots were constructed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA).

3 Results

Shading resulted in a larger leaf area in the two angiosperms, *E. urophylla* and *C. chinense*, but not in the conifer *P. macrophyllus* (Fig. 1). Shading also resulted in a higher specific leaf area in all three species. The *SD* of all three species decreased with shading, however, *SS* remained similar between sun- and shade-acclimated plants of *P. macrophyllus* and *C. chinense*. In *E. urophylla*, shading decreased the *SS* by over 25%. On leaf hydraulics, K_{leaf} was similar between sun and shade-acclimated plants of all three species when assessed under low PPFD (Table 1). When assessed under high PPFD, K_{leaf}

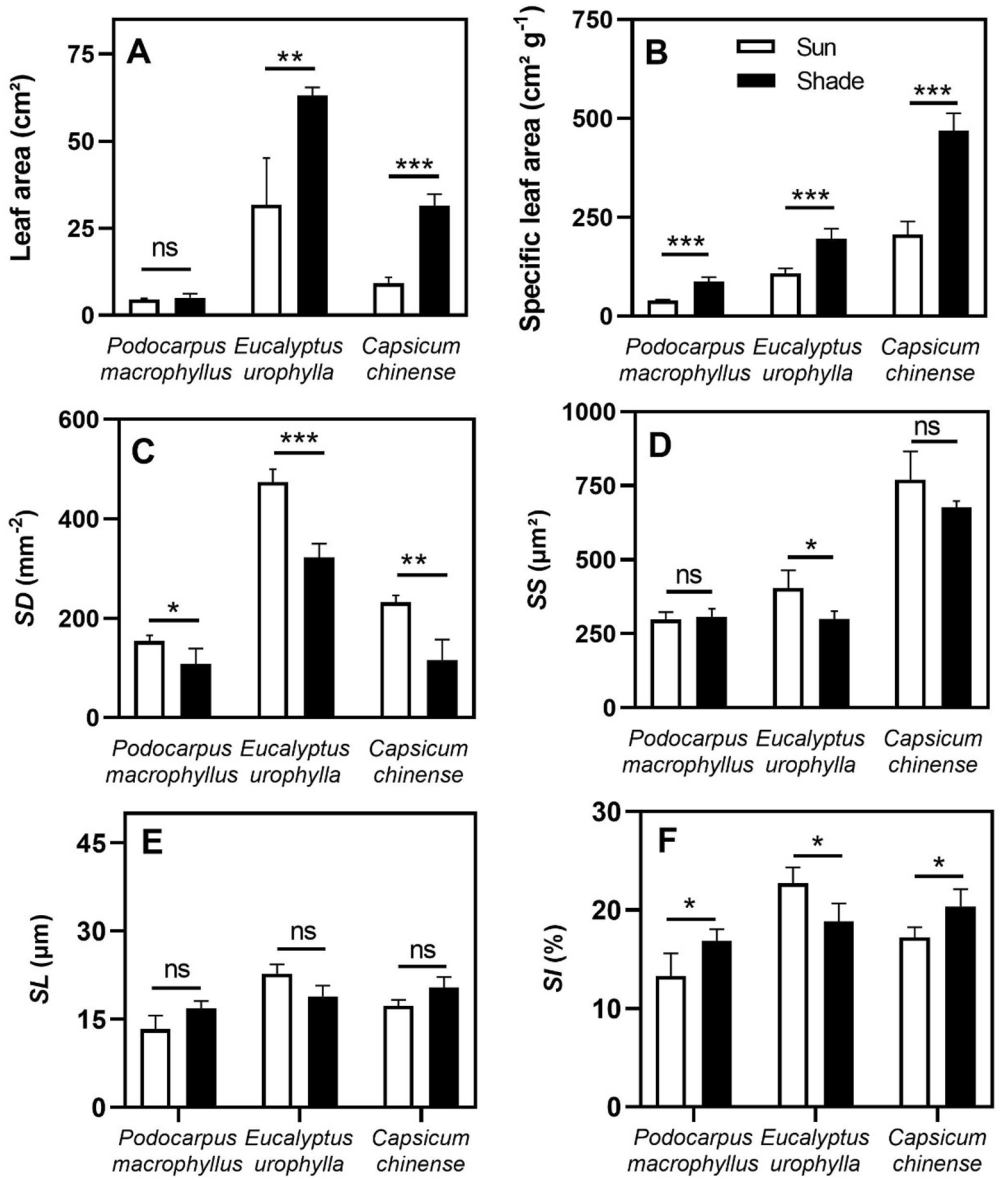

Fig. 1 **A** Leaf area, **B** specific leaf area, **C** stomatal density (*SD*), **D** stomatal size (*SS*), **E** stomatal length (*SL*), and **F** stomatal index (*SI*) of three woody species cultivated in either full sunlight (white bars) or shade (black bars). Data are means ± SD ($n = 4$). Results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

was higher in shade-acclimated leaves of *P. macrophyllus* and *E. urophylla*, and in sun-acclimated leaves of *C. chinense*.

The $t_{90opening}$ obtained from plants exposed to a step increase in light from dark to low PPFD was similar between sun- and shade-acclimated plants of all three species (Fig. 2A, C, E). The $t_{90opening}$ obtained when plants were exposed to a step increase in light from dark to high PPFD was also similar between sun- and shade-acclimated plants of *P. macrophyllus* and *E. urophylla*. The $t_{90closure}$ in response to low and high PPFD was also similar between sun- and shade-acclimated plants of *P. macrophyllus* and *E. urophylla* (Fig. 2B, D). Sun-acclimated plants of *C. chinense* exhibit a lower $t_{90closure}$ than their shade-acclimated counterparts from low PPDF to dark (Fig. 2E). Shade-acclimated plants of *C. chinense* were lost before stomatal curves at high PPFD could be performed.

Shade-acclimated *P. macrophyllus* exhibited a higher $Sl_{opening}$ after a step increase to low PPFD than sun-acclimated plants (Fig. 3). Conversely, sun-acclimated *E. urophylla* exhibited a higher $Sl_{opening}$ than shade-acclimated plants after a step increase to high PPFD. For stomatal closure, sun-acclimated *P. macrophyllus* exhibited a higher $Sl_{closure}$ from high PPFD to dark, and sun-acclimated *C. chinense*, a higher $Sl_{closure}$ from low PPFD to dark.

Neither the $t_{90closure}$ nor the $t_{90opening}$ correlated with maximum g_s (Fig. 4). However, while a wide range of $t_{90opening}$ was observed to occur in parallel with lower maximum g_s, only low values of $t_{90opening}$ were observed to occur in parallel with higher maximum g_s (Fig. 4C). Higher $Sl_{opening}$ and $Sl_{closure}$ correlated closely with higher maximum g_s (Fig. 4B and D).

4 Discussion

4.1 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 the area of leaves that developed under shade in the two angiosperms (Fig. 1). Such foliar area increase is likely a result of expansion in epidermal cell size, which is consistent with the considerable decreases in *SD*. These decreases are expected to occur due to a passive dilution mechanism as epidermal cells get larger (Carins Murphy et al. 2012, 2016). Leaves of the conifer *P. macrophyllus* acclimated to sun and shade exhibited similar areas and yet, the shade-acclimated leaves exhibited lower *SD* than their sun-acclimated counterparts (Fig. 1). Given the similar area of sun- and shade-acclimated leaves of *P. macrophyllus*, the lower *SD* found in shade-acclimated *P. macrophyllus* plants cannot be explained by the passive dilution of stomata due to increases in epidermal cell size. 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 consistently observed decrease in *SD* with shading, *SS* exhibited a more limited plasticity in response to growth PPFD (Fig. 1). Out of the

Table 1 Mean leaf hydraulic conductance (K_{leaf}) of three woody species cultivated at either full sunlight or shade conditions

Species	K_{leaf}			
	Sun 1000	Shade 1000	Sun 100	Shade 100
Podocarpus macrophyllus	2.8 ± 0.2	4.5 ± 1.3*	2.5 ± 0.5	2.8 ± 0.6^{ns}
Eucalyptus urophylla	11.9 ± 3.8	18.7 ± 1.1*	6.8 ± 1.5	4.4 ± 0.8^{ns}
Capsicum chinense	28.1 ± 1.6	15.3 ± 2.5**	8.0 ± 1.1	5.0 ± 3.5^{ns}

Results of unpaired Student *t*-tests between sun and shade under each light conditions are indicated (**$P < 0.01$; *$P < 0.05$; ns, $P > 0.05$)

The responses of sun and shade-acclimated plants were assessed at both low (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 ($n = 4$)

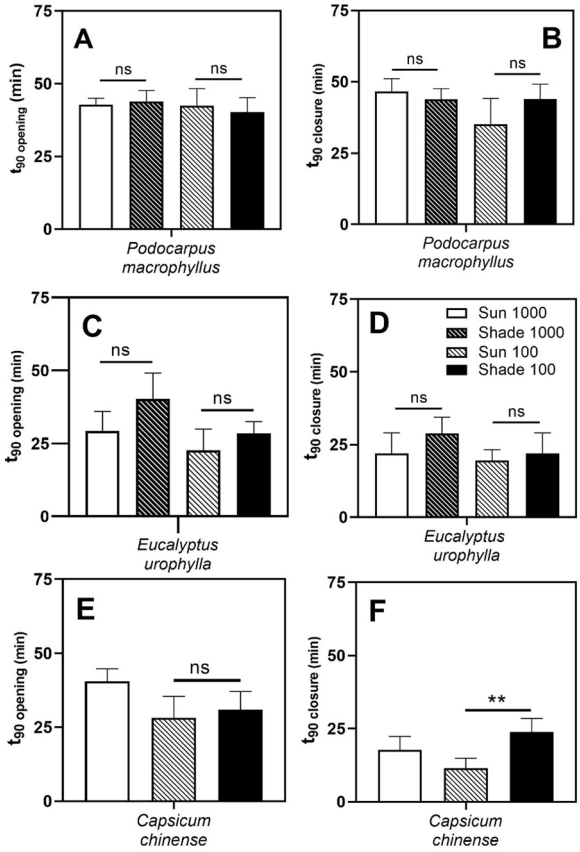

Fig. 2 **A**, **C**, **E** Time to reach 90% of maximum g_s increase ($t_{90opening}$) and **B**, **D**, **F** time to reach 90% of maximum g_s decrease ($t_{90closure}$) of three woody species cultivated at either full sunlight or shade conditions. The responses of sun (white background bars) and shade-acclimated plants (black background bars) were assessed at low (100 µmol photons m^{-2} s^{-1}) (black hatch to bars) or high photosynthetic photon flux density (PPFD) (1000 µmol photons m^{-2} s^{-1}) (white hatch to bars). Data are means ± SD ($n = 4$). The original curves are in Supplementary Figs. 3 and 4. Shade-acclimated plants of *C. chinense* were lost before stomatal curves at high PPFD could be performed. Results of unpaired Student *t*-tests are indicated above columns (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, $P > 0.05$)

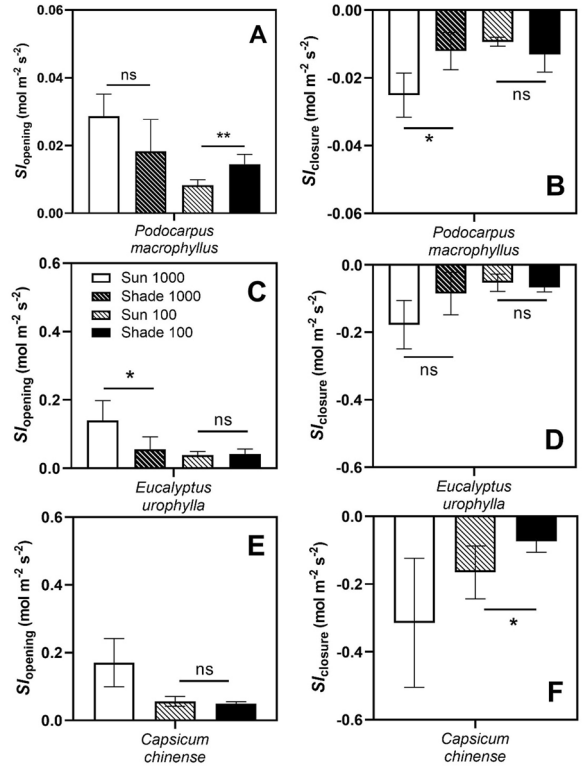

Fig. 3 **A**, **C**, **E** The maximal slope of g_s increase ($SI_{opening}$) and **B**, **D**, **F** the maximal slope of g_s decrease ($SI_{closure}$) of three woody species cultivated at either full sunlight or shade conditions. The responses of sun (white background bars) and shade-acclimated (black background bars) plants were assessed at both low (100 µmol photons m^{-2} s^{-1}) (black hatch to bars) and high photosynthetic photon flux density (PPFD) (1000 µmol photons m^{-2} s^{-1}) (white hatch to bars). Data are means ± SD ($n = 4$). The original curves are in Supplementary Figs. 3 and 4. Shade-acclimated plants of *C. chinense* were lost before stomatal curves at high PPFD could be performed. Results of unpaired Student *t*-tests are indicated above columns (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, $P > 0.05$)

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 in the other two species in response to PPFD. In addition, none of the three species exhibit significant plasticity in guard cell length in response to growth PPFD (Fig. 1). The insensitivity of *SS* to changes in PPFD has been previously documented in woody and herbaceous species (Carins Murphy et al. 2012, 2016; Martins et al. 2014; Kardiman and Ræbild 2018), while smaller stomata have also been observed in response to shading in herbaceous ferns and angiosperms (Carins Murphy et al. 2016, 2017). Along with results from the aforementioned studies, our findings demonstrate that the plasticity in *SS* in response to light is species-specific, yet the mechanisms underlining either the maintenance or reduction in *SS* remain elusive.

It is noteworthy that declines in *SD* with shading were either uncoupled from changes in *SS* (*P. macrophyllus* and *C. chinense*) or coupled with the decreases in *SS* (*E. urophylla*) (Fig. 1), adding to

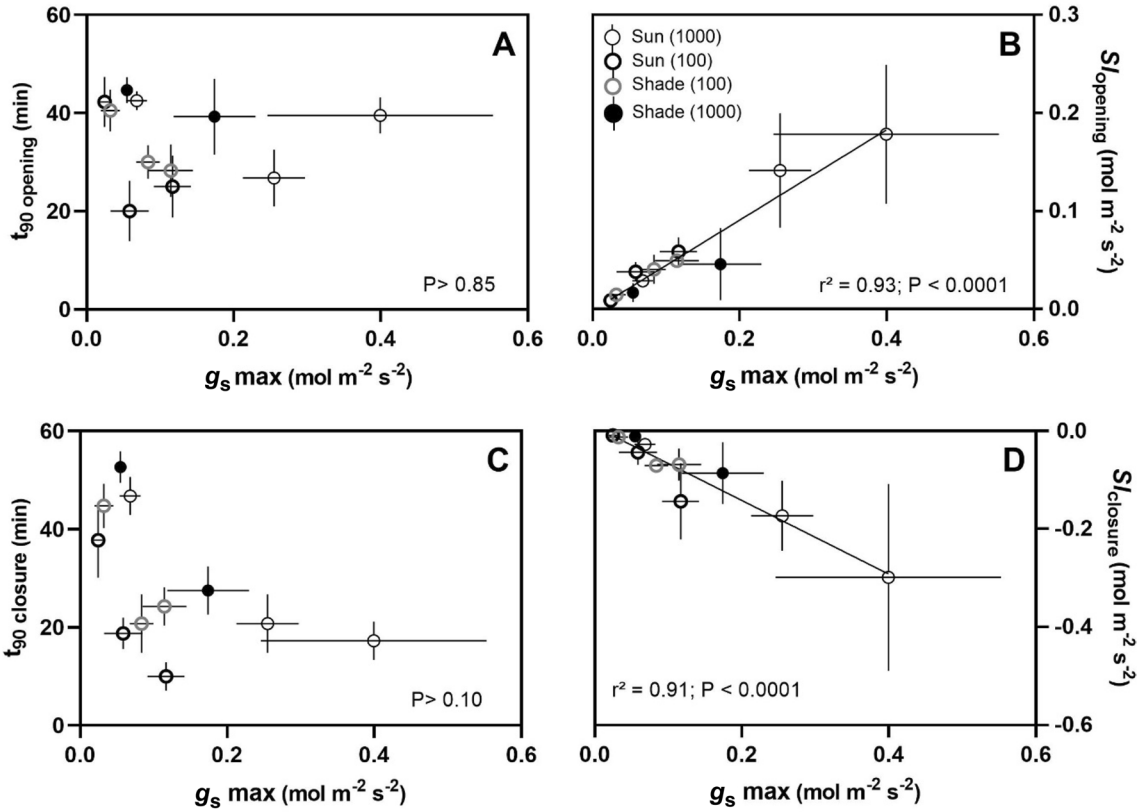

Fig. 4 Correlations between time to reach 90% of maximum g_s increase and decrease ($t_{90opening}$ and $t_{90closure}$) and maximum stomatal conductance (g_s) **A**, **C** and the maximal slope of g_s increase and decrease ($Sl_{opening}$ and $Sl_{closure}$) and maximum g_s **B**, **D** of three woody species cultivated at either full sunlight 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 photosynthetic photon flux density (PPFD) (1000 µmol photons m^{-2} s^{-1}). Maximum g_s were obtained from each of the original curves

a growing body of evidence that the interspecific alterations of both traits in response to light are independent (Supplementary Fig. 5) (Carins Murphy et al. 2012, 2016, 2017; Martins et al. 2014; Kardiman and Ræbild 2018). Particularly in 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) but agrees with studies that show no relationship between these two parameters across a range of species (McAusland et al. 2016). This finding reinforces the idea that changes in the size of epidermal cells occur independently of changes in guard cells, possibly because the final *SS* takes place well before the final expansion of the leaf (Schoch et al. 1980; Zwieniecki et al. 2004; Carins Murphy et al. 2012).

4.2 The t_{90} is better suited to compare stomatal speed of sun and shade leaves than Sl_{max}

The results obtained by the two methods selected to assess stomatal speed (t_{90} and Sl_{max}) did not entirely agree (Figs. 2 and 3). However, this is not entirely surprising given that the two methods are based on different traits of the stomatal opening and closure curves (Supplementary Fig. 2). The t_{90} simply accounts for the time to reach 90% of the maximum change in stomatal aperture (Drake et al. 2013; Deans et al. 2018). The Sl_{max} considers the changes in g_s over the time of linear response, which relies on the magnitude of maximum leaf gas exchange rates (Wong et al. 1979; Deans et al. 2018; Lawson et al. 2012; McAusland et al. 2016; Wall et al. 2022). The maximum gas exchange rate, in turn, relies on

stomatal function (dynamic aperture of the stomatal pore) and leaf anatomy (*SS* and *SD*). Our findings clearly support the strong dependence of Sl_{max} for stomatal opening and closure on maximum g_s through the thigh correlation between higher maximum g_s with higher modulus of $Sl_{opening}$ and $Sl_{closure}$ (Fig. 4). Conversely, the t_{90} was shown not to linearly rely on maximum leaf gas exchange rates (Fig. 4), confirming that this trait better suited to assess stomatal speed than *Sl* when comparing leaves with different gas exchange capacities (Deans et al. 2018). Interestingly, higher maximum g_s was never observed to occur in parallel to higher $t_{90closure}$, suggesting that leaves with higher transpiration rates always close their stomata faster, thus preventing excessive water loss and declines in water potential.

Leaves expanded in sun achieve higher *SD* than leaves expanded in shade (Fig. 1), and that can potentially result in different gas exchange capacities (Supplementary Table S2). Therefore, one can expect sun-acclimated leaves to achieve higher Sl_{max} than shade-acclimated leaves even if with identical changes in stomatal pore aperture. Therefore, results from t_{90} are more appropriate to assess changes in stomatal speed between sun- and shade-acclimated leaves. For instance, the higher $Sl_{opening}$ of sun-acclimated *E. urophylla* was observed without changes in $t_{90opening}$, such that the higher $Sl_{opening}$ is likely a result of the higher maximum g_s due to a higher *SD* (Supplementary Table S2) rather than a faster dynamic pore aperture (Figs. 2 and 3). A similar explanation is also likely true for the higher $Sl_{closure}$ decouple from changes in $t_{90closure}$ of sun-acclimated *P. macrophyllus* from high PPFD to dark (Figs. 2 and 3).

4.3 Leaves expanded either in sun or shade present similar t_{90}

The t_{90} was similar for nearly all species and conditions, except for *C. chinense* closure from low PPFD to dark, where shade-acclimated leaves took longer (higher t_{90}) to close their stomata in dark than sun-acclimated leaves (Fig. 2). This data was accompanied by a lower $Sl_{closure}$ in shade-over sun-acclimated leaves. It is noteworthy that stomata respond to a myriad of environmental stimuli—in terms of *SD* and aperture—but intra-specific variation in stomatal speed to light is limited. This result is especially surprising if we consider that sun-acclimated leaves achieve considerably higher *SD* than shade-acclimated ones. So far, studies on the environmental impact on the speed of stomatal movements are restricted to a few species (Gerardin et al. 2018; Kardiman and Ræbild 2018; Matthews et al. 2018) and contrasting results are found among species, throughout the time of the day, and between opening and closing responses. Overall, the speed of stomatal opening is less affected than the speed of stomatal closure. This pattern was also observed in the present study. However, neither of these studies could explain why variations across species are substantially larger than within species. Altogether, this demonstrates that our understanding on the acclimation of the speed of the stomatal conductance response is far from elucidated.

Faster stomatal kinetics have long been associated with greater *SD* and reduced *SS*. Our results on sun- and shade-leaves with very contrasting *SD*, however, challenge the idea that changes in *SD* itself can functionally explain the stomatal aperture speed of individual pores. Instead, the positive correlation between greater *SD* and stomatal speed can only be functionally explained when the method to assess stomatal speed takes into consideration the maximum leaf gas exchange associated with the higher *SD* (see discussion on Sl_{max} above). Conversely, changes in *SS* are more likely to result in changes in the behavior of individual pores. Smaller stomata present greater membrane surface area to volume ratio (Hetherington and Woodward 2003; Franks and Beerling 2009; Drake et al. 2013; Kardiman and Ræbild 2018), which facilitates the exchange of water and solutes between stomata and the cells around them (Lawson and Blatt 2014). The SS of sun- and shade-acclimated leaves of the three species evaluated in the study were similar, and different environmental or genetic manipulations are necessary to drive higher intra-specific changes in *SS*, so that changes in t_{90} can be tested in response to *SS*.

Additionally, a higher ability of leaves to move water through their tissues (i.e., K_{leaf}) could potentially lead to faster stomata. Higher PPFD—both dynamic and during leaf expansion—lead to higher K_{leaf} (Table 1), but that was not enough accompanied by changes in t_{90}. In addition to *SS*, *SD*, and K_{leaf}, it is possible that additional factors defining stomatal speed are essentially unaffected by PPFD during leaf expansion. Amongst these factors, we can cite

biochemical, structural, and molecular components of guard cells and vacuolar ionic dynamic transport (Medeiros et al. 2019; Lawson and Matthews 2020; Eyland et al. 2021). Previous studies have tried to elucidate how vacuolar channels and transporters command stomatal movements (Eisenach and Angeli 2017), and how changes in the activity of these channels impact the speed of stomatal opening and closure (Medeiros et al. 2016; Yang et al. 2020), but questions remain regarding the response of these components when exposed to contrasting light irradiance.

5 Conclusions

We demonstrate that the light condition at which woody species are grown results in a consistent variation in morphoanatomical and hydraulic properties in leaves. The time to reach 90% of the maximum change in g_s, however, is essentially insensitive to the changes in the leaf structure induced by the light condition during growth. These results suggest that stomatal speed has low phenotypic plasticity, likely being under stronger genetic regulation than other leaf and stomatal anatomical properties.

Author contributions AAC and TL designed the study; RSF, LAO, and AAC carried out the experiments; RSF and AAC analyzed the data and wrote the manuscript with revisions from FMD, SAMM, and TL; all authors read and approved the final version of the manuscript.

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Declarations

Conflict of interest The authors have no conflicts of interest to declare.

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