



Research paper

Leaf water potential-dependent leaflet closure contributes to legume leaves cool down and drought avoidance under diurnal drought stress

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Efficient thermoregulation under diurnal drought stress protects leaves from photosystem damage and water supply-demand imbalance, yet the cool effect and drought avoidance by leaflet closure have not been well understood. We investigated the cool effect and the drought avoidance of leaflet closure in legume species that survived in the semi-arid region facing seasonal and diurnal drought stress. The results showed that leaflet closure effectively cooled down legume leaves through a reduction of projected leaflet area and the cosine of the angle of incidence ($\cos i$). The leaflet closure was strongly dependent on leaf water potential (Ψ_{leaf}). In addition, by characterizing the sequence of key leaf drought response traits, we found leaflet closure occurred after stomatal closure and reduced transpiration rate but before hydraulic failure and turgor loss point (Ψ_{tlp}). The meta-analysis also showed that the leaflet closure and $\cos i$ decreased after the stomatal conductance declined but before midday. These results imply that Ψ_{leaf} -dependent leaflet closure as an alternative to transpiration for leaflet cooling down and as a protective drought avoidance strategy assisting sessile legume plants survival under drought stress.

Keywords: leaf temperature.

Introduction

Sunlight is the indispensable energy that plant leaves rely on to fix carbon dioxide into high-energy sugars through photosynthesis (Tilman 1988, Falster and Westoby 2003, Bittebiere et al. 2012, Craine and Dybzinski 2013). However, the intense light intensity is also harmful to leaves, for instance, even short-time exposure to the intensified light environment could cause a steep increase in leaf temperature, particularly in low wind conditions (Percy 1990, Vogel 2009). Drought stress combined with high temperature could damage the photosynthesis enzyme activities, alter cell differentiation and elongation and eventually result in leaf abscission and senescence (Vollenweider and Gunthardt-Goerg 2005, Potters et al. 2009, Mathur et al. 2014). A series of physiological regulations

and physical traits were evolved to cool leaves, such as enhanced transpiration taking away heat (Crawford et al. 2012, Pou et al. 2012), dense leaf hairs reflecting radiation (Lang et al. 1995, Ye et al. 2011) and small leaves convecting heat (Yates et al. 2010). The physical traits (e.g., dense leaf hairs and small leaf area) are the intrinsic leaf traits that seem unable to respond timely to the instantaneous diurnal drought stress. As such, the real-time diurnal leaf thermoregulation is mainly dependent on transpiration through regulating stomatal conductance. Although transpiration from opening stomata could cool leaves to a large extent, leaves have to close their stomata under prolonged heat stress to prohibit extensive water loss and which exposes the leaves to the great risk of desiccation (Schulze et al. 1973, Callwood et al. 2021).

The central reason for the ineffective leaf cool down through transpiration under drought stress is due to the loss of leaf water potential (Ψ_{leaf}). The decrease of Ψ_{leaf} would induce stomatal closure (Brodribb and McAdam 2011, 2017, McAdam and Brodribb 2014), cell turgor loss (Bartlett et al. 2014, McAdam and Brodribb 2016), embolism formation (Sperry 1986) and even plant mortality (Li et al. 2016). Low Ψ_{leaf} induced poor physiological performance would ultimately result in plants' survival and distribution (Feng et al. 2022). The Ψ_{leaf} at the 50% and 88% leaf hydraulic conductance decline is defined as $K_{\text{leaf}P_{50}}$ and $K_{\text{leaf}P_{88}}$, which is used to evaluate the drought tolerance of leaves, and once the Ψ_{leaf} is lower than $K_{\text{leaf}P_{50}}$ and $K_{\text{leaf}P_{88}}$ leaves would face great hydraulic failure risk (Martorell et al. 2015, Choat et al. 2018). The ability of plants to maintain diurnal leaf water status higher than $K_{\text{leaf}P_{50}}$ is thus critical for leaves' drought resistance as it is recognized as the bottleneck in the whole plant hydraulic transport system (Sack et al. 2003).

According to the physical rule that water moves toward lower water potential, leaf cells started to lose water and shrink when Ψ_{leaf} is lower than the leaf cell osmotic pressure (Scoffoni et al. 2014). Previous studies reported that leaf cells would shrink sharply when the Ψ_{leaf} is lower than turgor loss point (Ψ_{tlp}) and only slight cell volume changes when the Ψ_{leaf} is higher than Ψ_{tlp} (Meidner 1955, Kennedy and Booth 1958, Sancho-Knapik et al. 2010). In contrast, the leaf shrinkage was also observed when Ψ_{leaf} is higher than Ψ_{tlp} and which is closely associated with osmotic pressure at full turgor (π_0) (Fellows and Boyer 1978, Colpitts and Coleman 1997, Scoffoni et al. 2014). Nevertheless, the decline of Ψ_{leaf} induced cell shrinkage would ultimately lead to the leaf morphology change. A well-known example is that the legume species *Phaseolus acutifolius* change its leaflets paraheliotropically under dry and hot environments, which is triggered by the turgor loss of motor cells on one side of the pulvinus (Yu and Berg 1994). Due to Ψ_{leaf} being strongly driven by turgor pressure (Sancho-Knapik et al. 2010), the mechanical stimulation-induced leaflet closure in *P. acutifolius* is also related to the Ψ_{leaf} decrease (Yu and Berg 1994). The more convincing evidence of the linkage between leaflet closure and Ψ_{leaf} is that the leaflet closure in *Mimosa pudica* was also observed under drought stress (Patil and Vajjapurkar 2007). Furthermore, leaflet morphology changes under water-stressed conditions were observed in *Bauhinia brachycarpa* by closing its leaves and *Tarenna depauperata* by curling its leaves (Lin et al. 2017).

The apparent dependency of leaflet angle changes on Ψ_{leaf} under drought and heat stress raises the possibility that Ψ_{leaf} -dependent leaflet angle change might function on leaflets cooling down through the reduction of light interception leaf area. Abundant evidence support this standpoint. Leaflets narrowing their angles under the diurnal drought stress were observed in *Rhododendron maximum* (Nilsen 1985), *Atriplex hymenelytra*

(Mooney et al. 1977), *Lactuca serriola* (Werk and Ehleringer 1984), *Ocotea esmeraldana*, *Rodognaphalopsis discolor*, *Aspidosperma album*, *Retiniphyllum concolor*, *Remijia morilloi*, *Clusia* sp., *Macairea rufescens* (Medina et al. 1978) and *Glycine max* (Rakocevic et al. 2018). Theoretically, the Ψ_{leaf} -dependent leaflet closure would reduce the light interception leaf area and thereby reduce the leaf heat loading. Leaflet closure of legume species under diurnal drought stress has been observed worldwide (Berg and Heuchelin 1990, Amador-Vargas et al. 2014, Lin et al. 2017), which provides an excellent system to investigate the cooling effect of leaflet closure and the possible contribution to its drought avoidance. We hypothesized that the leaflet closure under diurnal drought stress cools leaves through the reduction of light intercepted leaf area and cosine of the angle of incidence ($\cos i$); the leaflet angle change is closely dependent on Ψ_{leaf} as previously reported; this fundamental mechanical leaflet closure under diurnal drought stress contributes to legume leaves drought avoidance.

To test the hypothesis, three legume species with different life forms (tree, herb and shrub) were examined (Table 1). Three legume species closed their leaflets in the clear hot summer midday was a general phenomenon in the field condition (Figure 1; see Figure S1 available as Supplementary data at *Tree Physiology* Online). We monitored the diurnal changes in leaf temperature and leaflet closure on the typical clear days in the mid-growing season (July 2021) to figure out to what extent the leaflet closure cools down the legume leaves. We investigated the driving role of Ψ_{leaf} on leaflet closure by analyzing the relationship between leaflet angle from the horizontal and Ψ_{leaf} during the leaflet closure process. Finally, to shed light on the ecological importance of the hypothesized Ψ_{leaf} -dependent leaflet closure on leaf drought avoidance under diurnal drought stress, we characterized the position of leaflet closure in the sequence of the key leaves' drought response traits of the species examined in this study and additionally conducted a meta-analysis on the time of day and the Ψ_{leaf} at 50% leaflet closure, $\cos i$ and stomatal conductance declined by extracting data from previous publications.

Materials and methods

Field study site and legume species examined

The field experiments were conducted in Linze Inland River Basin Research Station (100° N 8' S, 39° E, 21' W) located in the west of Gansu province, Northern China. The study site experienced extreme seasonal drought stress with a mean annual precipitation of 97.2 mm, evaporation of 2385 mm, temperature of 8.9 °C, wind speed of 3.2 m/s and photosynthetic active radiation (PAR) of 9382 mol m⁻² (<http://lzdc.cern.ac.cn/>). In the mid-growing season, plants experienced the highest PAR, air temperature and water vapor pressure, but relatively low precipitation (<5 mm) and wind speed in the field (see

Table 1. Three legume species height, leaf anatomic traits, leaf turgor loss point and leaf water potential at predawn (Ψ_{pre}) and midday (Ψ_{mid}).

Legumes species	Life form	Height (m)	Leaf size (cm ²)	Leaf thickness (mm)	Ψ_{pre} (–MPa)	Ψ_{mid} (–MPa)
<i>R. pseudoacacia</i>	Tree	7.50 ± 0.45	4.22 ± 0.43	0.23 ± 0.02	0.35 ± 0.1	1.80 ± 0.3
<i>A. dahuricus</i>	Herb	0.35 ± 0.08	0.28 ± 0.03	0.25 ± 0.02	0.25 ± 0.1	1.35 ± 0.2
<i>C. korshinskii</i>	Shrub	1.85 ± 0.35	0.23 ± 0.04	0.27 ± 0.03	0.40 ± 0.1	2.40 ± 0.3

Figure S2 available as Supplementary data at *Tree Physiology* Online). In July 2021, three widely planted legume species with different growth forms (tree: *Robinia pseudoacacia*; herb: *Astragalus dahuricus*; shrub: *Caragana korshinskii*) that closed their leaflets under diurnal drought stress in the hot mid-summer were examined (Table 1).

Diurnal changes of leaf temperature, leaflet angle, $\cos i$ and surrounding environmental variables

We measured the diurnal leaf temperature changes on a typical clear day in six mature sun-exposed leaves from three individuals every 10 min starting predawn at 6:00 a.m. and finished sunset at 21:30 p.m. To precisely measure the legume leaves temperature, leaf temperature was measured by both infrared thermometer (MS-WITO2, MEET International Ltd, Hong Kong, China) and K type thermocouple thermometer (Proster Digital Thermocouple Temperature Thermometer, Proster Trading Ltd, Hong Kong, China), in which the difference between the two thermometers was <0.3 °C. The mean value of the two thermometers was used. Due to the leaf temperature being varied across the leaf surface (Saudreau et al. 2017), we used a unified method by measuring the temperature in the middle of the leaf between the mid-vein and leaf margin to represent the leaf's temperature.

In parallel with leaf temperature measurement, we recorded the diurnal changes of leaflet angle from the horizontal, air temperature, light intensity and wind speed across the whole measurement. Three conventional ways used to document leaflet closure are the leaflet angle from the horizontal (ranging from 0° to 90°, where 0° is horizontal and 90° is vertical) the angle between leaflets (ranging from 0° to 180°, where 180° is horizontal and 0° is vertical) (Berg and Heuchelin 1990, Huang et al. 2014) and the cosine of the angle of incidence ($\cos i$) (ranging from 0 to 1, where 0 means leaflet is parallel to the sun's rays, 1 means leaflet is perpendicular to the sun's rays) (Ehleringer and Forseth 1980). The leaflet angle from the horizontal was measured by an inclinometer parallel to the adaxial leaflet surface and $\cos i$ was calculated according to the angle between the leaflet and perpendicular to the sun's direct rays as previously described (Ehleringer and Forseth 1980, Prichard and Forseth 1988). Air temperature and light intensity were measured by the gas thermometer and light detector in a gas exchange system (Li6400,

Li-Cor Inc., Lincoln, NE, USA). Wind speed was measured by an anemometer (RA320; Jinru trading limited, Dongguan, China). To get the projected leaflet area differences of the opened and closed leaflets, we took the top-down leaflet photography with a horizontal white background paper (Canon EOS 5D, Tokyo, Japan). The images of the projected leaflet area were processed and calculated by ImageJ software (Abramoff et al. 2004).

Leaf water potential

Ψ_{leaf} in predawn (5:00–5:30 a.m.) (Ψ_{pd}) and midday (12:00–14:00 p.m.) (Ψ_{mid}) was determined by the Scholander-type pressure chamber (Model 1515D; PMS Instrument Company, Corvallis, OR, USA) in July 2021. In each species, 6–10 leaves from three individuals were sampled in the predawn and midday and were put into a black plastic bag with a wet towel separately. The wet towel could keep the bag with some humidity and thus stop the leaf water loss. The towel and leaf were put on the different sides of the bag to avoid direct attachment. The sampled leaflet in the bag was equilibrated for at least 20 min before the Ψ_{leaf} measurement, to investigate the relationship between Ψ_{leaf} and leaflet angle from the horizontal. On three clear days, during the leaflet closure process, the leaflet angle was first measured by an inclinometer, then the leaflet was cut and the Ψ_{leaf} was measured as described above. A total of ~15 leaves from three individuals of each species were measured to construct the relationship between Ψ_{leaf} and leaflet angle.

Gas exchange measurements

Leaf-level stomatal conductance (g_s) and transpiration rate (E) were measured as Ψ_{leaf} decreased from predawn to midday with a portable photosynthesis system (Li6400, Li-Cor Inc., Lincoln, NE, USA) in situ. For the species with a small leaf area that could not fully fill the leaf chamber, we calibrated the leaf area-based gas exchange values by multiplying the ratio of chamber area to leaf area (chamber area/leaf area). To get the natural diurnal response of g_s and E to Ψ_{leaf} , the field measurements were conducted on three typical clear days. CO₂ concentration, light and temperature within the leaf chamber were the same as the ambient conditions. The rate of airflow through the leaf chamber was set to 500 $\mu\text{mol s}^{-1}$. g_s and E were expressed on a projected leaflet area basis. Shortly, the sun-exposed mature leaves from at least two individuals of each

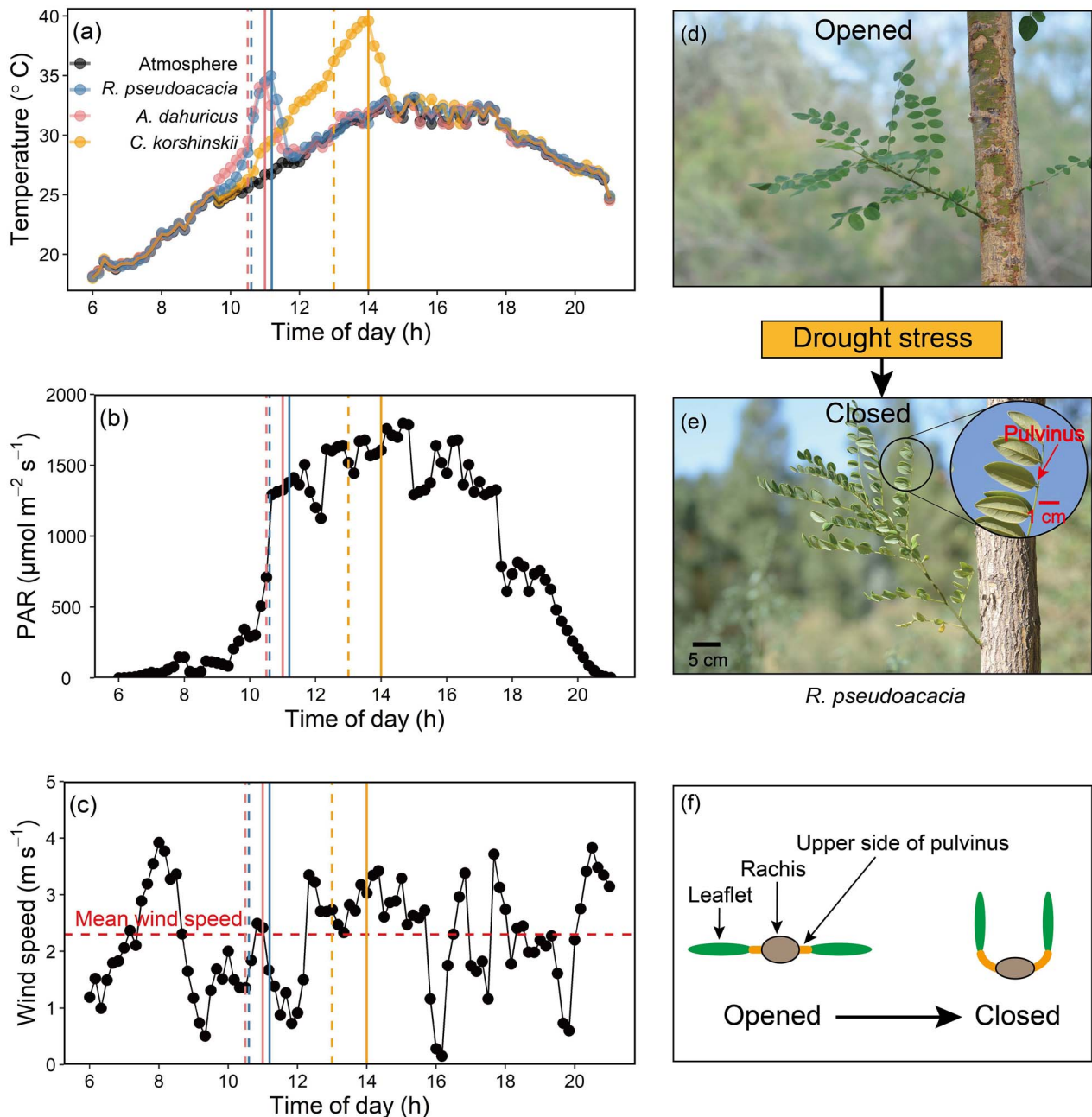


Figure 1. The diurnal changes of leaflet closure, leaf temperature, air temperature, solar radiation and wind speed on a typical clear summer day. (a) The diurnal changes of leaf temperature, air temperature and leaflet closure. The leaf temperature of three legume species increased sharply when the leaflet kept open and followed a rapid cool down after leaflet closure. (b) and (c) The diurnal changes of PAR and wind speed. The colored dashed lines represent the time when the legume leaves started to close and the solid lines represent the time the leaves completely closed, different colors represent different species as the legends show. The red dashed line in (c) represents the mean wind speed. (d) and (e) The leaflet angle change of *R. pseudoacacia* from open to closure under acute heat stress. The red arrow in (e) indicates the pulvinus where the leaf bent occurred. The scale bar is shown in (e) and the subplot. (f) The model plot of the leaflet from open as (d) shows to closure as (e) shows.

species were selected to measure g_s and E as Ψ_{leaf} decreased from predawn to midday. Once the stabilized gas exchange values were logged, the leaves were excised immediately and Ψ_{leaf} was measured as described above. Finally, g_s and E were plotted against Ψ_{leaf} . The functional response of g_s and E to Ψ_{leaf} was determined by the optimum functions (see Statistics section).

Pressure–volume curves

Pressure–volume curves were constructed by the bench drying method (Tyree and Hammel 1972). Three sun-exposed mature shoots were sampled from three individuals of each species the day before pressure–volume curves construction. The sampled shoots were put into a bucket with a terminal end cut two times under the ultra-pure water and transported to the laboratory

in 30 min. When the branches rehydrated overnight ($\Psi_{\text{leaf}} > 0.3$ MPa), 6–8 mature and fully expanded leaves' areas were scanned. To minimize the impact of oversaturation on the shape of the pressure–volume curves ('plateau effect') (Dichio et al. 2003, Meinzer et al. 2014), the portion of shoots that had been underwater was removed before the determination, which resulted in a gradual but not steep initial decline of $1/\Psi_{\text{leaf}}$ as relative leaf water content decreased. These leaves' fresh weight and Ψ_{leaf} were measured repeatedly as the leaves dehydrated on a bench. The osmotic pressure at full turgor (π_0) and turgor loss point (π_{tlp}) were calculated following the standard method described by Sack et al. (2011).

Leaf hydraulic conductance

Leaf hydraulic conductance (K_{leaf}) under different water statuses was measured by examining the kinetics of Ψ_{leaf} relaxation in rehydrating leaves following a previous publication (Brodribb and Holbrook 2003), which is based on the formula:

$$k_{\text{leaf}} = C_{\text{leaf}} \ln(\Psi_i / \Psi_f) / t$$

where Ψ_i is the initial Ψ_{leaf} (MPa), Ψ_f is the final Ψ_{leaf} (MPa) and t is the duration of rehydration time (s). Briefly, 3–5 rehydrated branches ($\Psi_{\text{leaf}} > -0.3$ MPa) of each species were sampled for K_{leaf} measurement. The shoots were allowed to desiccate slowly in the shade. The branches were put into black bags for Ψ_{leaf} equilibration when Ψ_{leaf} decreased every 0.3–0.5 MPa. When Ψ_{leaf} was homogenous in the whole shoot (the differences between two neighboring leaves smaller than 0.3 MPa), the mean Ψ_{leaf} of two neighboring leaves was recorded as Ψ_i . The leaflet in the middle was cut under ultra-pure water to rehydrate for t seconds. The time for rehydration is decided by the Ψ_i , the lower Ψ_i with longer rehydration t (t ranged from 2 to 250 s). For each species, C_{leaf} was calculated from the slopes of the pressure–volume curves. Because of the cell walls' elasticity, C_{leaf} pre- and post- π_{tlp} are so different. C_{leaf} was thus calculated from the slopes of the relationship between relative leaf water content and Ψ_{leaf} on either side of the π_{tlp} and normalized by leaf area. Leaf hydraulic vulnerability curves were constructed based on the functional response of K_{leaf} to Ψ_{leaf} by the sigmoidal ($y = \frac{a}{1+e^{-bx}}$) function.

Meta-data collection

The time of day and Ψ_{leaf} at which the 50% leaflet closed, g_s declined, and $\cos i$ decreased were compiled from previously published sources (see Table S1 available as Supplementary data at *Tree Physiology* Online). We conducted literature searches using the keywords 'leaflet closure', 'heliotropic leaf movement', 'leaflet angle', 'stomatal conductance' and 'drought stress' from Web of Science, Google Scholar and China National Knowledge Infrastructure (<http://www.cnki.net>). We extracted the data from the studies with drought treatment or under

control conditions. When values of the time of day and Ψ_{leaf} at which the 50% leaflet closed, g_s declined, and $\cos i$ were not reported in numerical form, they were extracted from published graphs of vulnerability curves by image processing software Engauge Digitizer (<http://markummittchell.github.io/engauge-digitizer>).

Statistics

The projected leaflet area and anatomical differences between opened and closed leaves were evaluated by one-way ANOVAs (Duncan). The optimum curves of leaflet angle, g_s , E and K_{leaf} with Ψ_{leaf} were selected from four functions by *optim* function in R following the standard method described previously (Scoffoni et al. 2011). The four functions are linear ($y = ax + b$), sigmoidal ($y = \frac{a}{1+e^{-bx}}$), logistic ($y = \frac{a}{1+(\frac{x}{x_0})^b}$) and exponential ($y = y_0 + ae^{-bx}$), respectively. The y in the expressions represents either the leaflet angle, g_s , E or K_{leaf} ; the x in the expressions represents Ψ_{leaf} . The fitted function with the lowest Akaike Information Criterion was selected as the best-fitted function to construct the fitted curve. Significant level (P -value) and r square were used to assess the goodness of the fit. The Ψ_{leaf} at the 50% declines of maximum g_s , E and K_{leaf} were calculated from the best-fitted curves. All the statistical analyses in the study were conducted in R programming (R Core Team 2020).

Results

Diurnal changes of leaf temperature, leaflet closure, $\cos i$ and projected leaflet area under diurnal drought stress

On the clear summer day of the hot and dry mid-summer in the field (see Figure S2 available as Supplementary data at *Tree Physiology* Online), the peak air temperature reached 32 °C and was accompanied by PAR ($> 1250 \mu\text{mol m}^{-2} \text{s}^{-1}$) and low wind speed (mean wind speed of 2.3 m s^{-1}) (Figure 1). The leaf temperature started to deviate from the air temperature at ~10:00 a.m. when PAR increased rapidly from 500 to 1200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. With the continuous deviation of leaf temperature to air temperature closing to noon, the leaflets closed rapidly within half an hour to 1 hour (Figure 1a). After the leaflets' closure, the leaf temperature of three legume species decreased rapidly from the peak and got close to the air temperature regardless of the remaining high air temperature, intensified light intensity and low wind speed (Figure 1a–c).

Detailed observation revealed that the leaflet closure under diurnal drought stress in the legume species was due to the upward bend of the pulvinus (Figure 1d–f; see Figure S1 available as Supplementary data at *Tree Physiology* Online). The projected leaflet areas significantly decreased after the leaflets closed in three legume species (Figure 2). Specifically, the mean projected leaflet area decreased by 93%, 80% and 77% in

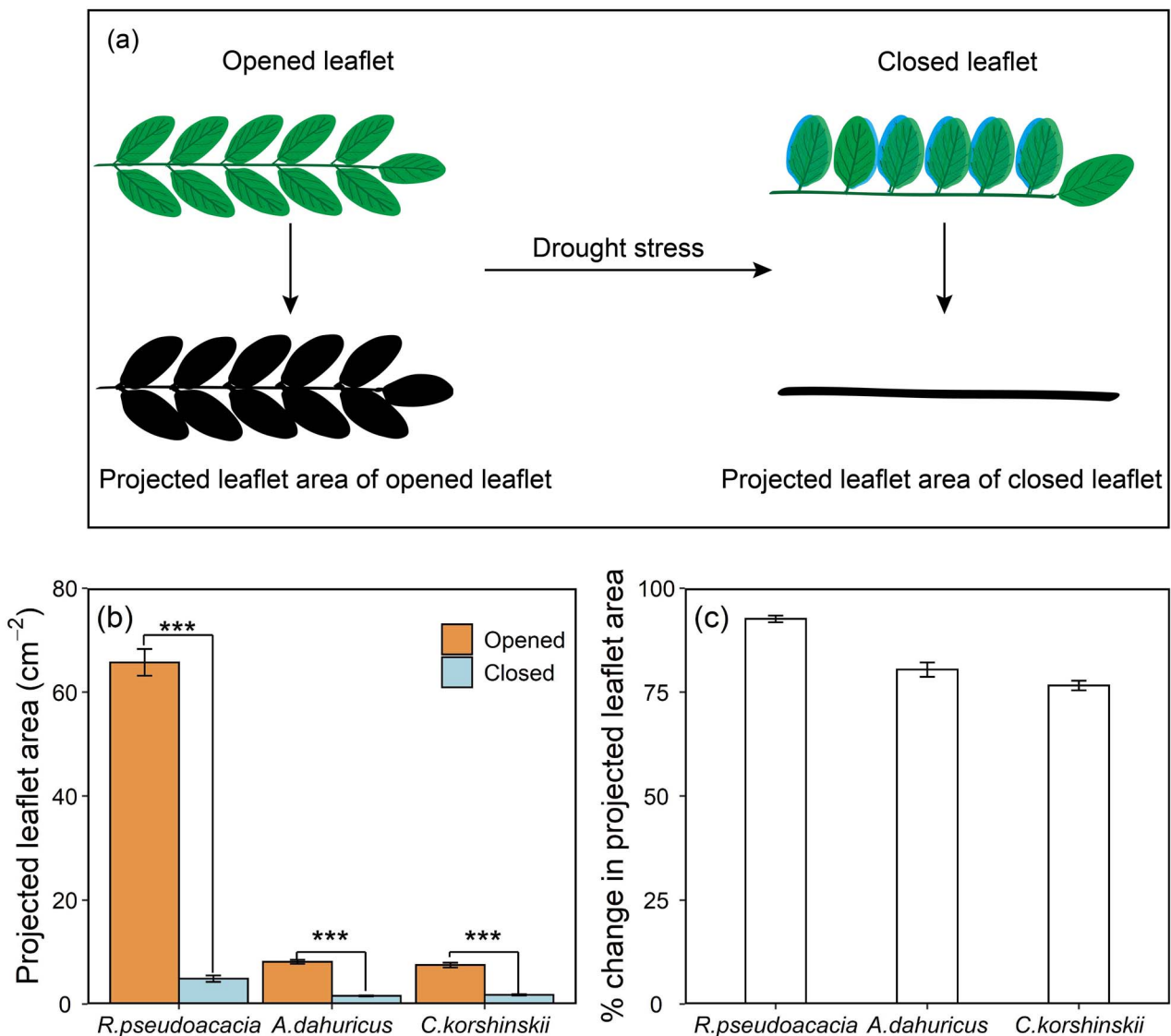


Figure 2. The projected leaflet area of opened and closed leaflets of three legume species: (a) the model plot of projected leaflet area from open to closure under acute heat stress; (b) the projected leaflet areas of three legume species were significantly different in open and closed leaves. The colors represent the opened and closed legume leaves as the legend shows. (c) The percentage change of the projected leaflet area after the leaflet closure. The standard error bar is shown in each volume. $n = 6$; $***P < 0.001$.

R. pseudoacacia, *A. dahuricus* and *C. korshinskii*, respectively. Similar to decreased projected leaflet area in closed leaflets, $\cos i$ of the leaflets declined remarkably after the leaflet closed and closed to 0 at the midday (Figure 3a, c and e). Such a great decrease of projected leaflet area and $\cos i$ of the closed leaflets greatly reduced the solar radiation interception and thereby reduced the heat load and eventually cooled down legume leaflets.

Leaf water potential and leaflet angle

The legume species experienced similar less negative water potential at predawn ($\Psi_{pre} > -0.4$ MPa) but experienced very distinct water potential at midday (Ψ_{mid} ranged from -1.35 to -2.4 MPa) (Table 1). In three legume species, we observed a strong dependency of leaflet closure on Ψ_{leaf} in the field

(Figure 3b, d and f). The leaves were kept open in relatively high Ψ_{leaf} . Only when reaching the critical Ψ_{leaf} , the leaflet angle from the horizontal started to increase linearly with Ψ_{leaf} until the leaflets were completely closed. The Ψ_{leaf} intervals for leaflet closure for *R. pseudoacacia*, *A. dahuricus* and *C. korshinskii* were -0.95 to -1.40 , -0.85 to -1.20 and -1.45 to -2.20 MPa, respectively. In addition, the critical Ψ_{leaf} that the leaf started to close was very close to but a little more negative than π_0 (Figure 3).

Leaflet closure in the sequence of leaf drought response traits

The Ψ_{leaf} at 50% declines in stomatal conductance ($g_s P_{50}$), transpiration rate (EP_{50}) and $K_{leaf} P_{50}$ were calculated from the vulnerability curves (Figure 4). $g_s P_{50}$ and EP_{50} in three legume

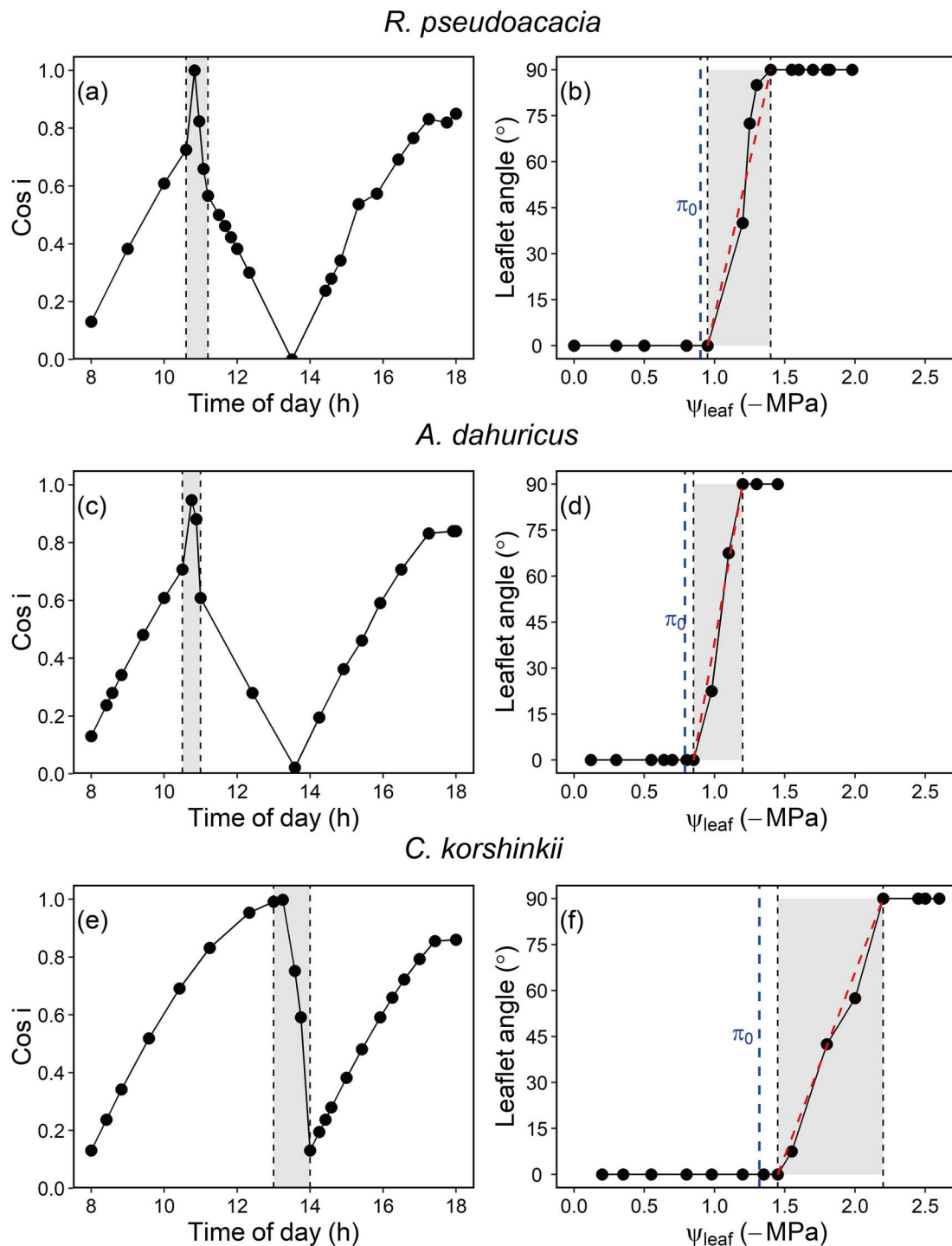


Figure 3. The relationships of legume leaflet angle from the horizontal with leaf water potential (Ψ_{leaf}) and the cosine of the angle of incidence (cos i) over the day in the field: (a) and (b) represent the relationship of leaf angle from the horizontal with Ψ_{leaf} and cosine of the angle of incidence during the day for *R. pseudoacacia*; (c) and (d) for *A. dahuricus*; (e) and (f) for *C. korshinskii*, respectively. The shallow areas represent the critical Ψ_{leaf} and time intervals where leaf closed. In three legume species, the leaf angle changed linearly at the critical Ψ_{leaf} interval as the red dashed lines show (for curved lines in (a), (c) and (e): $r^2 = 0.94$, $P < 0.01$; $r^2 = 0.95$, $P < 0.01$; $r^2 = 0.93$, $P < 0.01$). The leaf closure of three legume species greatly reduced the cosine of the angle of incidence in the hot midday. The blue line represents the osmotic water potential at full turgor (π_0).

species were similar in each species and higher than -1 MPa (Figure 4a–f). In comparison, $K_{\text{leaf}}P_{50}$ was more negative than g_sP_{50} and EP_{50} (Figure 4g–i). By characterizing the sequence

of key leaf drought response traits, we found a typical sequence of leaf drought response traits that are expected to prohibit the leaves from severe damage during diurnal drought stress

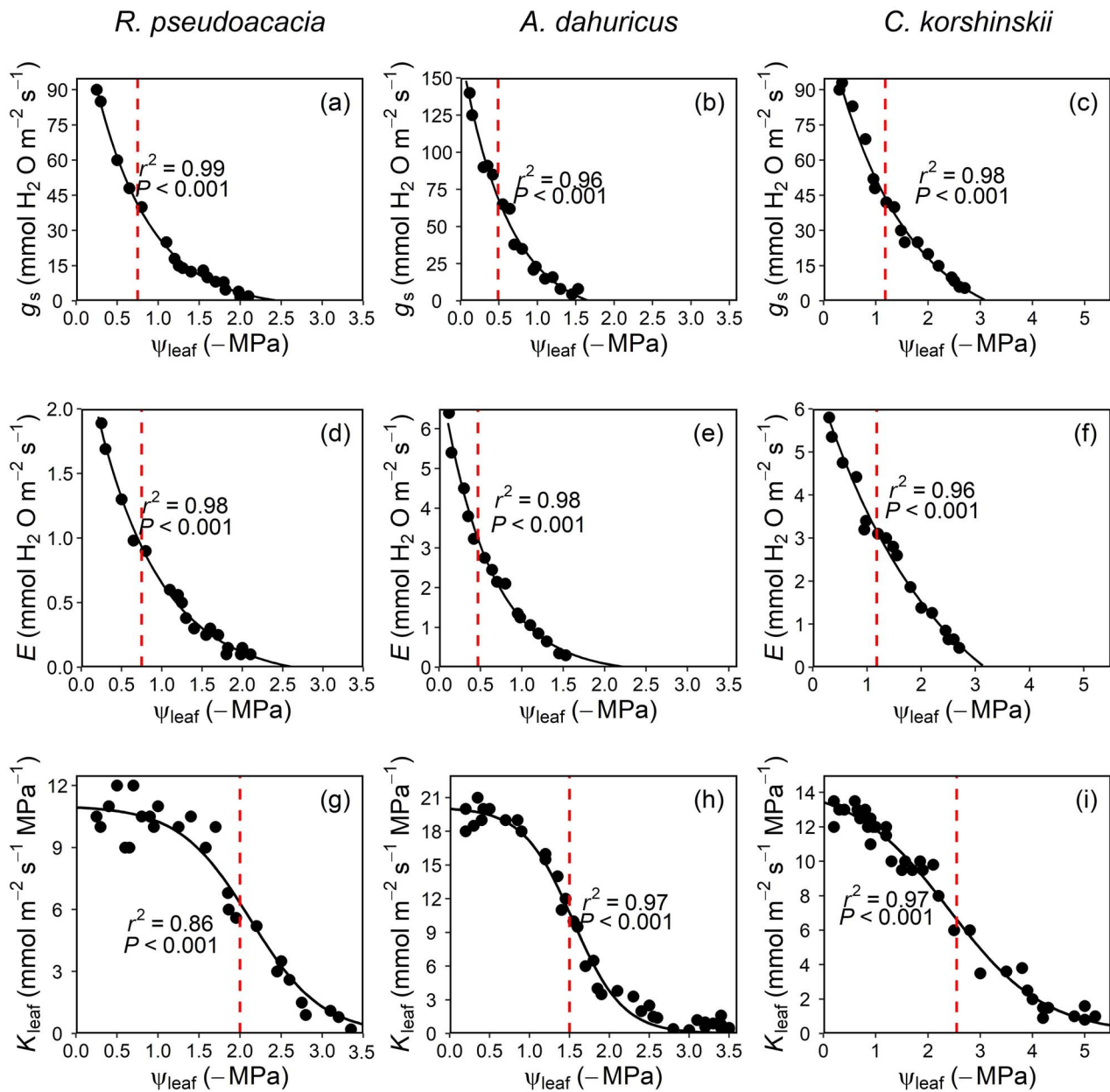


Figure 4. The vulnerability curves of stomatal conductance, transpiration rate and leaf hydraulic conductivity: (a), (d) and (g) the leaf water potential at the 50% declines of stomatal conductance, transpiration rate and leaf hydraulic conductivity were -0.75 , -0.75 and -2 MPa for *R. pseudoacacia*; (b), (e) and (h) -0.49 , -0.47 and -1.5 MPa for *A. dahuricus*; (c), (f) and (i) -1.18 , -1.18 and -2.55 MPa for *C. korshinskii*, respectively, and these are indicated by the red dashed lines. Ψ_{leaf} , leaf water potential; g_s , stomatal conductance; E , transpiration rate and K_{leaf} , leaf hydraulic conductivity.

(Figure 5). In which, $g_s P_{50}$ and $E P_{50}$ occurred at the least negative Ψ_{leaf} , followed by π_0 , leaflet closure, $K_{leaf} P_{50}$ and Ψ_{tlf} . In addition, the $K_{leaf} P_{50}$ of three legume species was only a little higher than Ψ_{mid} , exhibiting a narrow hydraulic safety margin (Figure 5).

The meta-analysis also supported the general sequence of drought response traits in legume species. Consistent with the Ψ_{leaf} induced the 50% leaflet closed was lower than the $g_s P_{50}$ in this study. Meta-analysis showed that the time of day and the Ψ_{leaf} at 50% leaflet closure and $\cos i$ decline occurred after the

50% stomatal conductance decreased (Figure 6). Furthermore, both in the present study and previous studies, the time of day that leaflets closed all occurred before noon (Figures 1 and 6a), implying that the Ψ_{leaf} induced leaf closure higher than the Ψ_{mid} .

Discussion

The findings in the present study provide strong support for the hypothesis that Ψ_{leaf} -dependent leaflet closure under diurnal

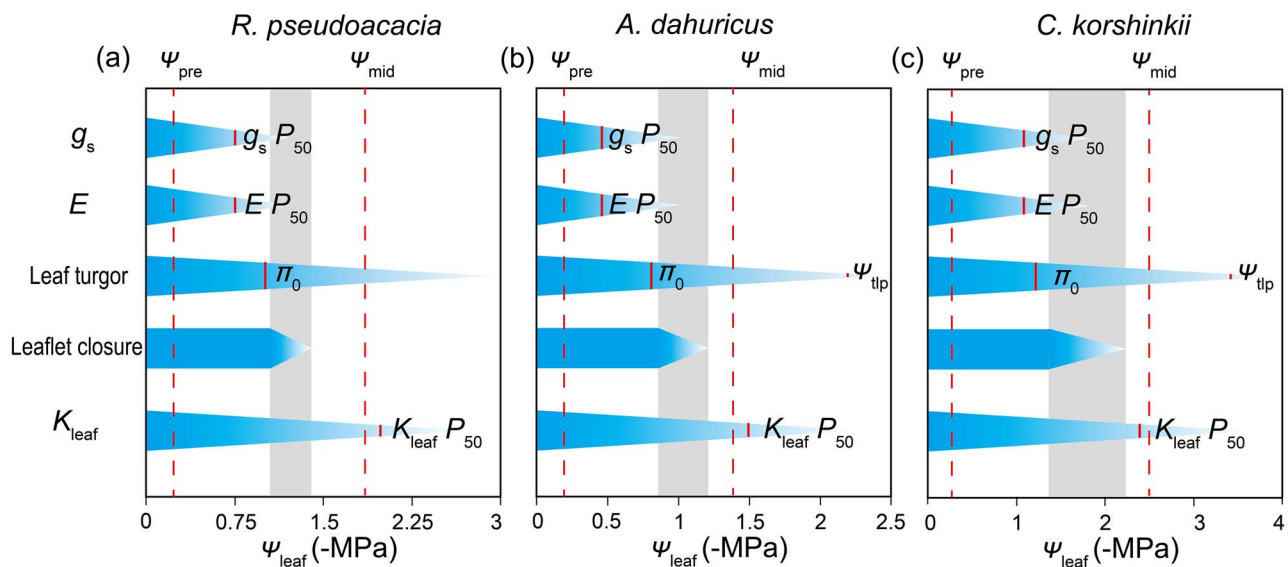


Figure 5. Sequence of legume leaves drought response traits under acute heat stress: (a), (b) and (c) show the sequence of drought response traits of *R. pseudoacacia*, *A. dahuricus* and *C. korshinskii*, respectively. In three legume species, with the Ψ_{leaf} decreased, leaf drought response traits sequenced by $g_s P_{50}$, $E P_{50}$, π_0 , leaflet closure, $K_{\text{leaf}} P_{50}$ and Ψ_{tip} . The shaded area represents the Ψ_{leaf} interval while the leaf was closed. Ψ_{leaf} , leaf water potential; Ψ_{pre} , leaf water potential in predawn; Ψ_{mid} , leaf water potential at midday; g_s , stomatal conductance; E , transpiration rate; K_{leaf} , leaf hydraulic conductance; π_0 , the osmotic potential at full turgor; Ψ_{tip} , turgor loss point; $g_s P_{50}$, $E P_{50}$ and $K_{\text{leaf}} P_{50}$ represent the leaf water potential at 50% declines of maximum g_s , E and K_{leaf} , respectively.

drought stress effectively cooled down legume leaves through the reduction of light interception leaflet area. The key position of leaflet closure after stomatal closure and before hydraulic failure emphasizes the mechanical leaflet closure as an alternative to transpiration for leaflet cooling down and as a drought avoidance strategy for legume plants' survival under diurnal drought stress. These remarkable observations raise a thoroughly novel perspective on the process that active leaflet movement for plant stress avoidance upon the sessile lifespan with recurrent droughts.

In legume species, we observed that leaflet closure at hot summer midday is critical for leaflet cool down. Before the leaflet closure, the maximum leaf temperature reached $\sim 35\text{--}40^\circ\text{C}$ and which greatly deviated from the air temperature and exposed the leaflet to high desiccation risk (Mathur et al. 2014). Although maintaining high stomatal conductance under drought stress was thought as one of the most efficient leaf cooling means (McClellan et al. 2011, Prasad et al. 2017, Urban et al. 2017), in this study, we found the legume species stomatal was sensitive to water stress and lost conductance at relatively less negative water status ($g_s P_{50} > -1$ MPa). It suggests that legume leaflets could not cool down through transpiration when reached relatively low Ψ_{leaf} . Alternatively, we found the leaflet temperature decreased rapidly after the leaflets closed vertically, implying leaflet closure plays a critical role in leaflet cool down under low Ψ_{leaf} conditions. The finding was consistent with the observation in *B. brachycarpa* that closed its leaves under diurnal drought stress regardless of the cooling effect of leaflet

closure (Lin et al. 2017). In three legume species, leaflet closure caused the projected leaflet area to decrease by more than 75% (Figure 2) and the rapid decrease of $\cos i$ (Figure 3). We thus argue that leaflet closure cools legume leaves through the decreased projected leaflet area and $\cos i$ through reducing the light intercepting leaflet area. The idea supported the theory that leaflet angle is the determinant factor of light interception and thus as the selection pressure of leaf structure and function (Walter 1979, Smith et al. 1998).

A strong dependency of leaflet closure on Ψ_{leaf} was found. In the critical Ψ_{leaf} intervals, the leaflet angle closed linearly with Ψ_{leaf} . It indicated that legume leaflet closure is closely dependent on Ψ_{leaf} . In stark contrast to the mechanical stimulation that induced leaflet closure in *M. pudica* (Burgert and Fratzl 2009, Amador-Vargas et al. 2014), we found no evidence that mechanical stimulation could induce the leaflet closure in the species in this study and also many other plants (Braum 2005). Furthermore, leaflet closure seems also independent of the circadian rhythm because the legume leaflets were kept open all day long on cloudy days. The light intensity and air temperature might also act as the possible drivers of leaflet closure because the leaflet closure occurred under high light intensity and high air temperature conditions. The possible light and temperature signals related to leaflet closure need to be investigated in future studies through physiological and molecular tools. In this study, we argued that the leaflet closure was closely dependent on Ψ_{leaf} and which was driven by the shrinkage of the motor cells on the pulvinus where the leaf bent

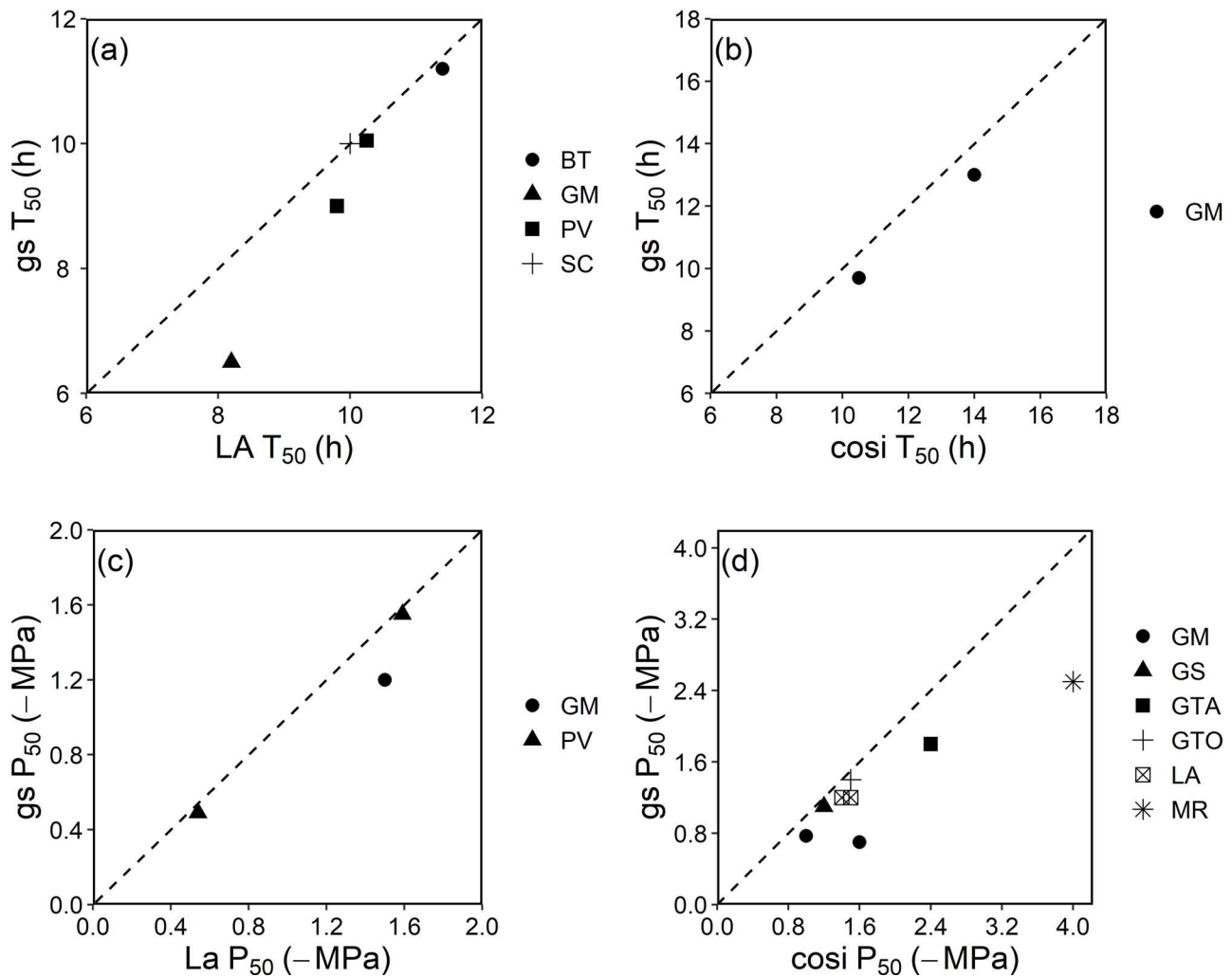


Figure 6. Meta-analysis shows that the time of day and the leaf water potential at 50% leaflet closure and cosine of the angle of incidence decline occurred after the 50% stomatal conductance decreased: (a) the time of day at 50% leaflet angle closed ($LA T_{50}$) and stomatal conductance decreased ($g_s T_{50}$); (b) the time of day at 50% cosine of the angle of incidence declined ($cosi T_{50}$) and $g_s T_{50}$; (c) the leaf water potential at 50% leaf angle closed ($La P_{50}$) and stomatal conductance decreased ($g_s P_{50}$) and (d) the leaf water potential at 50% cosine of the angle of incidence declined ($cosi P_{50}$) and $g_s P_{50}$. The dashed line in the plots represents a 1:1 line. The samples in the right of each plot represent the species codes and the detailed species information and meta-data are supplemented in Table S1 available as Supplementary data at *Tree Physiology Online*.

occurred just like *M. pudica* (Niinemets et al. 2004, Hejnowicz and Barthlott 2005). Observations of the legume species' pulvinus and osmotic traits strongly supported our hypothesis. An obvious upward bend of the pulvinus in closed leaves was observed (Figure 1), which implies that the pulvinus motor cells shrunk and stretched the leaflet closure as previously reported (Yu and Berg 1994). Furthermore, we found that the leaf started to close only when the Ψ_{leaf} was slightly lower than π_0 . It suggests that the motor cells shrunk were closely coordinated with π_0 . In contrast to the motor cells, the other mesophyll cells seem not shrunk at all because the Ψ_{leaf} induced leaflet closure is much higher than Ψ_{tip} (Figure 5) (Kennedy and Booth 1958, Sancho-Knapik et al. 2010). It means that the motor cells are more sensitive to water loss than mesophyll cells, just like the guard cells on the epidermis. Considering the inter-specific

differences, leaflets' closure of the tree species *R. pseudoacacia* and the herb species *A. dahuricus* were more sensitive to Ψ_{leaf} loss compared with the shrub species *C. korshinskii* (Figure 3). It implies that *C. korshinskii* is more tolerant to heat and drought stress than *R. pseudoacacia* and *A. dahuricus*, which is consistent with the lower $g_s P_{50}$, EP_{50} and $K_{leaf} P_{50}$ in *C. korshinskii* than the other two species (Figure 4).

Although the Ψ_{leaf} -dependent leaflet closure appears to occupy a critical role in the functioning of leaf cool down, the importance of leaf movement under diurnal drought stress for species drought avoidance remains to be seen. Given the sensitive leaflet closure in response to Ψ_{leaf} in the hot summer midday, it seems possible that leaf movement is a fundamental mechanical mechanism protecting leaves from overheating and even hydraulic failure. The idea was supported by characterizing

the sequence of the leaves' drought response traits. According to the drought response sequence, the leaflet closure occurred exactly after the stomatal closure, and before the $K_{\text{leaf}P_{50}}$ and Ψ_{tip} (Figure 5). It suggests that leaflet closure is an alternative to transpiration protecting leaves from overheating in face of drought and heat stress. The meta-analysis also supported the idea that the leaf closure and the cosine of the angle of incidence decreased after the stomatal conductance declined and before midday (Figure 6). Additionally, leaflet closure occurred before the $K_{\text{leaf}P_{50}}$, Ψ_{tip} and Ψ_{mid} might also function on protecting the leaves from hydraulic failure due to the narrow legumes leaves safety margins (Figure 5). Leaflet closure thereby might be a 'random' factor that alters the uncertain hydraulic trade-off (Gleason et al. 2016, van der Sande et al. 2019) by affecting the leaf hydraulic safety but awaits to be further explored in future works.

The Ψ_{leaf} induced leaflet closure in the hot summer midday recorded in the legume species ranged from -0.85 to -1.45 MPa, suggesting that the critical Ψ_{leaf} for leaflet closure varied across species. Unsurprisingly, such Ψ_{leaf} range in the midday has been recorded in a great number of species across different climates over the globe (Choat et al. 2012), but the leaflet closure is not generally observed. The species that could not close their leaflets under heat stress might link to their habitat environment, leaf shape, leaf size and texture. For instance, species that survived in shed environments are not exposed to high light environments even over the whole lifespan, thereby, the leaflet closure to avoid heat stress for these species seems unnecessary. Also, the species that lived in a stable environment such as the tropics with sufficient soil water supply could easily cool their leaves through transpiration by sufficient soil water supply instead of energy-cost leaflet closure. It is consistent with the observations of leaflet closure often reported in water deficit areas (Medina et al. 1978), as well as in this study. Furthermore, the mechanical force needed for leaflet closure might also be an important constraint. According to the mechanic's rules, leaflet closure needs to force the leaf fold by the motor cells shrunk in the pulvinus, in the case of the big and thick leaves, the shrinkage of motor cells in the pulvinus might not able to produce sufficient force to completely fold the leaves. This to some extent explains why the leaflet closure has not been observed in the species with big and thick leaves. Even if low Ψ_{leaf} could cause the slight leaflet angle change in these species, slight movements are hard to capture and the influences on the projected leaflet area are very tiny. Consistently, the legume species observed in the present study indeed with a small leaf size of fewer than 5 cm^2 and a low leaf thickness of $<0.3 \text{ mm}$ (Table 1). Regardless of the critical constraints on leaf movement still awaiting to be explored, based on the clear link between Ψ_{leaf} and leaflet angle in legume species, we proposed the Ψ_{leaf} -dependent leaflet closure under diurnal drought stress is a drought avoidance mechanism for legume species with thin and small leaves that habitat in hot-dry environments.

Our results provided clear evidence that the Ψ_{leaf} -dependent leaflet closure reduced projected leaflet area as the fundamental mechanical mechanism for leaf cool down and drought avoidance. It suggests that, regardless of the sessile organism, the active leaf movement could also help plants avoid environmental stress. Unlike the leaflet closure for heat avoidance in this study, the other forms of leaflet movements such as leaf curling and shrinkage under poor water status were also widely observed (Werk and Ehleringer 1984, Lin et al. 2017). The leaflet curling and shrinkage could also significantly reduce the light interception area, and thus might cool leaves just as leaflet closure. It needs to be noted that, no matter in our study or the other studies, the active leaf movements are always observed under low-speed or still wind conditions (Mooney et al. 1977, Nilsen 1985, Lin et al. 2017). Under light airflow conditions, the slow gas exchange between leaf and atmosphere seems unable to cool leaflets in an efficient way (Schymanski and Or 2016). In stark contrast to our observation that the active leaflet movement cools legume leaves under low-speed wind conditions, the passive leaflet movement was also observed under high wind speed conditions (Wilson 1980, Vollsinger et al. 2005, Vogel 2009). The morphology change under high-speed wind protects the leaves from mechanical damage driven by the tearing strength (Vogel 2009). The strategic dichotomy is of paramount importance because the active and passive leaf movements must yield different cost and defense trade-offs. Investigations into the costs associated with leaf movements under different stress conditions are required to understand how sessile plants survive successfully over the various ecological and physiological challenges.

Conclusions

In conclusion, our results demonstrated that Ψ_{leaf} dependent leaflet closure in legumes under diurnal heat and drought stresses helped leaflet cool down through the reduction of sun-exposed leaflet area. The leaflet closure occurred after stomatal closure and reduced transpiration rate but before hydraulic failure and turgor loss point implying that Ψ_{leaf} dependent leaflet closure as an alternative to transpiration for leaflet cooling down. This exquisite mechanical adjustment of leaflet morphology in response to leaf water status provides the insight that the active leaflet closure assists sessile plants to avoid diurnal heat and drought stresses.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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Authors' contributions

W.Z. and X.F. provided the idea and designed the experiment. X.F., L.Z. and Q.T. conducted the investigation, processed data and prepared the figures. X.F. and W.Z. wrote and reviewed the manuscript.

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