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ORIGINAL PAPER

Factors controlling plasticity of leaf morphology in *Robinia pseudoacacia L.* II: the impact of water stress on leaf morphology of seedlings grown in a controlled environment chamber

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Abstract
• ***Context*** The cause of morphological plasticity of leaves within the crowns of tall trees still debated. Whether it is driven by irradiance or hydraulic constraints is inconclusive. In a previous study, we hypothesized that water stress caused between-site and within-tree morphological variability in mature *Robinia* trees.
• ***Aims*** To test this hypothesis, we designed an experiment to analyze the effect of long-term water stress on leaf growth of *Robinia* seedlings in a controlled environment.
• ***Methods*** Two treatments were performed: well-watered (midday water potential, Ψ_w=−0.45 MPa) and water-stressed (Ψ_w=−1.0 Mpa), which resulted in significant differences in physiology, relative growth rate, and the temporal progress of leaf growth.
• ***Results*** Variation of leaf cell sizes among treatments was comparable to the variability previously observed in the field. However, values of leaf density and leaf mass per unit area tended to be lower in our controlled experiments than in the field, which may reflect differences between mature leaves of juvenile and adult trees.
• ***Conclusions*** Our tentative conclusion is that leaf water stress may be the primary factor controlling morphological changes observed in the field, but further experiments are needed to document the relative importance of irradiance.

Keywords Leaf growth · Water stress · Temporal evolution · Tree height

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Contribution of the co-authors Yanxiang ZHANG: did the experimental work, data analysis, and first draft.
Maria Alejandra EQUIZA: provided training in methods and oversaw experimental work.
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1 Introduction

The plasticity of leaf morphology within the canopy profile of trees has received increased attention (Sack et al. 2006; Cavaleri et al. 2010; Oldham et al. 2010). The debate recently has been over the relevance of irradiance versus height (hydraulic constraint) as the primary determinant of the plasticity (Ellsworth and Reich 1993; Sack et al. 2006; Marshall and Monserud 2003; England and Attiwill 2006; Cavaleri et al. 2010; Oldham et al. 2010). Since almost a century ago, the study of leaf morphology and its response to environmental factors such as irradiance, vapor pressure deficits, temperature, and relative humidity has been studied

(Hanson 1917). Previous studies have shown that leaves developed under higher irradiance levels have longer and stacked palisade cells and larger and more abundant mesophyll cells, which results in increased leaf thickness and leaf dry weight per area (LMA) (Ellsworth and Reich 1993; Hikosaka et al. 1994). Based on these previous studies, the plasticity of leaf morphology along tree height has been assumed to be mainly irradiance-driven (Ellsworth and Reich 1993; Sack et al. 2006). However, increasing recent evidence is suggesting that leaf morphological plasticity in tree canopies is much more strongly related to hydrostatic constraints than to irradiance (Marshall and Monserud 2003; Koch et al. 2004; England and Attiwill 2006; Cavaleri et al. 2010; Oldham et al. 2010). Water potential declines due to hydrostatic (gravity-induced) and hydrodynamic resistance in tall trees (e.g., Tyree and Zimmermann 2002; Woodruff et al. 2004), which reduces the turgor pressure necessary for cell expansion and, as a consequence, affects the size and morphological characteristics of the leaf.

Changes in both cell division and cell expansion can underlie the leaf morphological plasticity observed along a tree's vertical gradient. In dicotyledonous species, two developmental phases have been identified during leaf expansion (Lecoeur et al. 1995; Pereyra-Irujo et al. 2008; Granier and Tardieu 2009). In the first phase, leaf size can be described by an exponential function with time and leaf growth coordinates cell division and tissue expansion, absolute growth rate (*GR*) is slow, and relative growth rate (*RGR*) is high (Pothig and Sussex 1985). In the second phase, cell division slows down and individual cell area increases resulting in tissue expansion at a high *GR* (Granier and Tardieu 1998). Previous studies on different dicotyledonous crop species (Lecoeur et al. 1995; Granier and Tardieu 1999, 2009) have shown that water stress reduces both GR and RGR resulting in smaller final leaf area and higher leaf LMA.

Our experimental data from adult *Robinia* trees in the field (Paper I of this series) showed a significant morphological plasticity of leaves along the vertical tree canopy, with leaves on the top of the crown being smaller and thicker than those at the bottom. Our data also suggested that leaf morphology and anatomy were more associated with hydraulic constraints than with irradiance, because of significant differences in morphology between wet and dry sites at comparable heights. In the present study, two levels of water stress imposed on seedlings in controlled environments were used to mimic hydraulic constraints at the top of *Robinia* trees. This kind of experimental approach, which we have previously applied to study height-related hydraulic constraints in a conifer species (Zhang et al. 2011), has been used to isolate the effect of water potential gradients from other environmental gradients that would normally occur across the canopy of a tree (e.g., irradiance, temperature, and relative humidity). The present study will focus on the effect of long-term water stress on the growth of one dicotyledonous woody species—*Robinia pseudoacacia* L., with the aim to assess if mild water stress in *Robinia* seedlings will result in morpho-anatomical changes similar to those previously observed in adult trees <16 m tall in the field.

In this study, we analyzed the effects of water stress on temporal changes of leaf growth, cell expansion, and division during the development of *Robinia* leaves experiencing stable, long-term mild water stress compared with well-watered controls. We also compared the results in this study and the results in the field in paper I of this series and confirmed the effects of hydraulic constraints on leaf morphological plasticity along tree height.

2 Materials and methods

Experiments were conducted in a growth chamber at the University of Alberta from April to July 2010. The daily photoperiod was 18 h (from 600 to 2400 hours) with artificial light provided by 400-W xenon lamps. Maximum photosynthetic photon flux density was 350 μmol m^{-2} s^{-1}. Temperature was kept at 22°C/18°C (day/night) and relative humidity at 75%.

Twenty-four *R. pseudoacacia L.* seedlings were grown in 2-L pots filled with a potting mix composed of peat moss (55–65%), perlite, dolomitic limestone, and gypsum (Sunshine® LA4 mix, Sun Grow Horticulture Canada Ltd.). Lateral stems were removed as soon as they became visible so all measurements were taken on upper terminal shoots. During the first month, all seedlings were kept well watered. Thereafter, two watering regimes were imposed for 90 days: (1) well-watered (midday Ψ_w was around −0.45 MPa), (2) water-stressed (midday Ψ_w was kept around −1.00 MPa) (Table 1). The difference of water potential between these two treatments was kept around 0.5 MPa, which is the difference of water potential at the top and bottom of *Robinia* trees in the field based on our experimental data in paper I of this series.

Table 1 Summary of water potential Ψ_w (MPa), net photosynthesis (μmol·CO_2·m^{-2} s^{-1}), and stomatal conductance g_s (mol·m^{-2} s^{-1}) in the two treatments: control and drought

Variables	Control	Drought	*P* value
Water potential	−0.483±0.0919a	−1.011±0.021b	<0.001
Net photosynthesis	7.363±0.382a	1.652±0.133b	<0.001
Stomatal conductance	0.186±0.0414a	0.021±0.00108b	<0.001

Different letters behind the values indicate significant difference between treatments ($P<0.05$). Values are means ± SE. Each mean value was the average value of 12 plants per treatment

Twelve seedlings per treatment were used. The surface of the containers of all water-stressed plants was covered with aluminum paper to limit evaporation from the soil. Plants were 100–110 cm tall and with about 8 mm basal-stem diameter at the start of the treatments.

During the first week of the two treatments, midday stomatal conductance (g_s) was measured daily using a portable open-flow photosynthesis system (LI-6400, Li-Cor, Inc) equipped with a LI-6400B red/blue LED light source. Once leaves acclimated to the conditions within the chamber (CO_2 386 μmol mol^{-1}, air temperature 21±0.15°C, and VPD 1.3±0.3 kPa, PPFD 500 μmol photon m^{-2} s^{-1}), data were logged every 30 s during a 5-min period. The average of the ten measurements obtained for each plant was used for data analysis. Midday leaf water potential Ψ_w was measured every other day using a pressure bomb (Soil Moisture Equipment Corp., Santa Barbara, USA) until it reached the selected targets. As soon as the target stress levels were reached, the weight was recorded for each pot in the late afternoon. Targets were subsequently maintained by adding water to each pot on a daily basis at 1800 hours to restore the previous day's weight. On typical days, the amount of water added was 130 g per 2-L pot for each drought plant. Midday Ψ_w and g_s were assessed once per week until the end of the experiment to confirm that target stress level was properly maintained.

2.1 Growth measurements

Since the effect of water deficit depends largely on the timing of the treatment, relative to the development of the leaf (Granier and Tardieu 1999), all the samples were selected from the new leaves budded from the terminal meristem after all the treatments were at the target stress levels. Measurements of length of the main petiole of the compound leaf and length and width of the fourth leaflet from each compound leaf were started when leaves were about 1% of final leaf length and leaflet area, respectively. Preliminary experiments indicated that 1% final size (leaf length and leaflet area) occurred when leaves were 2.75 mm and 10.4 mm^2 for controls and 2.00 mm and 6.05 mm^2 for stressed plants, respectively. Leaf length was defined as the distance from a mark made at the base of the petiole to the base of the terminal leaflet of the odd pinnate compound leaves of Robinia. Daily measurements of leaf length were performed with a ruler while leaflet length and width were measured with a digital caliper until leaf growth ceased. A highly significant linear relationship (r^2>0.99, n=192, P<0.0001) was established between length×width and leaflet area, which was applicable to any leaf regardless of leaf number, leaf age, or treatment. The fourth pair of leaflets from the base of the compound leaves and three consecutive leaves per plant was selected for the leaf growth measurements. Based on our preliminary experiment (not shown), leaf final size was not related to leaf sequence on the stem when tree height was taller than 0.8 m. The duration of both leaf and leaflet growth was defined as the number of days from the moment of 1% of final leaf length and leaflet area to the moment of 95% of final leaf length and leaflet area was reached. *GR* was calculated as the slope of the relationship between length (L) and time (t) in two consecutive measurements, and *RGR* was calculated as the slope of the relationship between the logarithm of length (L) and time (t): $GR=[d(L)/dt]$ and $RGR=[d(\ln(L))/dt]$, and similar equations are applied for leaflet area (Pereyra-Irujo et al. 2008).

Six to eight leaflets (the fourth leaflet from the base of leaf) per plant were chosen for laboratory measurement of leaflet area and leaflet dry weight. An image of each leaflet was scanned by a scanner, and then leaflet area was calculated using the software ImageJ (Image Processing and Analysis in Java; http://rsb.info.nih.gov/ij/). Thereafter, these leaflets were dried in an oven at 70°C for 48 h and then weighed to determine dry weight and *LMA* (*LMA* = dry weight/leaflet area). Leaflet density was equated to *LMA*/leaflet thickness. Leaf thickness was not measured every time LMA was calculated so thickness measurements were sometimes interpolated from plots of thickness verus % final leaf length by using a polynomial fit.

2.2 Leaf anatomical measurements

Leaflet paraffin sections were made at 5%, 10%, 25%, 50%, and 100% of final leaf length. For each growth point, two leaflets per plant, 12 plants per treatment, were selected for sections. All the subsamples for sections were picked from the middle base (about 6×6 mm) of each fresh leaflet. Samples were fixed in FAA (95% ethanol/glacial acetic acid/formalin/distilled water=10:1:2:7), dehydrated in ethanol series and embedded in paraffin. Cross sections, 6-μm thick, were stained with safranin O-fast green and mounted in DPX. Images were obtained with a digital camera (Infinity1-5C, Regent Instruments Inc., Quebec, Canada) mounted on a microscope (Axioskop 40, Zeiss, Jena, Germany). Leaflet thickness, first-layer palisade mesophyll cells (located immediately under upper epidermis), cell length and width, and upper and lower epidermal cell width and thickness were calculated using the software ImageJ.

2.3 Data analysis

Data were analyzed with one-way ANOVA. Differences of mean values between treatments were separated by Tukey's HSD Test with 95% confidence level.

3 Results

3.1 Leaf physiology and growth

The water stress imposed in this experiment induced a significant reduction in net photosynthesis (77% reduction compared to controls) and stomatal conductance (88% reduction) (Table 1).

Leaflet number per compound leaf was similar for controls and stressed plants, 21.58±0.48 and 21.23±0.29 for control and drought, respectively (P=0.918; data not shown). Leaf and leaflet growth were significantly affected by water stress (Fig. 1). The GR and RGR were significantly reduced under water stress. Water stress affected the duration of growth phases. The first phase (1% to 25% final length) included days 1 to 15 in controls and 1 to 20 in stressed plants; the second phase (25% to 95% final length) included days 16 to 30 in controls and 21 to 40 in stressed plants.

3.2 *LMA* and leaflet density

During leaf growth, *LMA* and leaflet density changed significantly at all phases of growth expressed as the leaf-length ratio (the ratio of growing leaflet area to final leaflet area, Fig. 2). *LMA* increased markedly before 25% of final leaf length and then dropped a bit during leaf rapid growth (25% to 95% of final leaf length) (Fig. 2a). Leaflet density increased rapidly until a peak at around 10% of the final leaf length, and then it dropped at a constant rate (10% to 95% of final leaf length) (Fig. 2b). At maturity, both *LMA* and density increased (100% of final leaf length). During leaf growth, *LMA* was always greater in water-stressed than in well-watered plants (Fig. 2a). Similar to *LMA*, leaf density was higher for stressed plants during leaf development (Fig. 2b).

3.3 Leaflet anatomical structure

During leaf growth, leaflet palisade mesophyll and epidermal cell size showed significant variation between treatments (Figs. 3 and 4). Leaflet thickness increased with leaf age, but it was always greater in water-stressed plants than in controls (P<0.001; Fig. 4a). The length of first-layer palisade mesophyll cells increased with leaf age regardless of the treatment. At maturity, water-stressed plants had longer palisade mesophyll cells than controlled plants, although before maturity, the length was smaller (P<0.001; Fig. 4b). The width of the first-layer palisade cells did not show variation with time, and it was always smaller in the water-stressed plants (P<0.001; Fig. 4b). Epidermal cell width and thickness increased with leaf age. While epidermal cell width was smaller under water stress (P<0.001; Fig. 4c), epidermal cell thickness did not exhibit a consistent trend between treatments during leaf growth (Fig. 4d).

During leaf growth, epidermal and palisade mesophyll cell number per leaf cross-sectional area decreased with leaf age (Table 2). At 5% of final leaf length, epidermal and palisade mesophyll cell number per cross-sectional area were smaller under water stress than controls (P<0.001). At 10% and 25% of final leaf length, cell number per cross

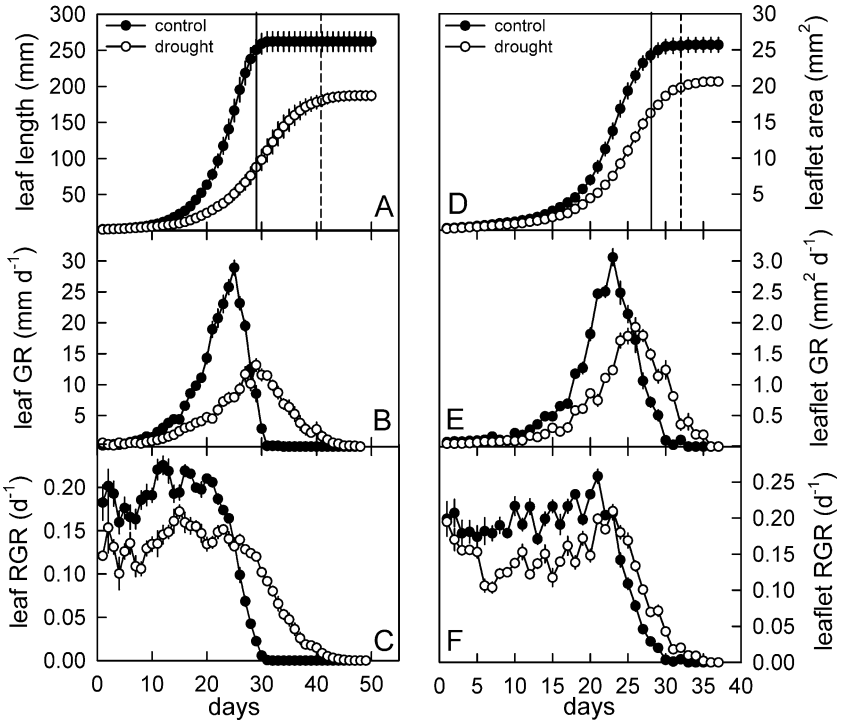

Fig. 1 Temporal changes in leaf length (**a**), leaf growth rate (*GR*) (**b**), leaf relative growth rate (*RGR*) (**c**), leaflet area (**d**), leaflet *GR* (**e**) and leaflet *RGR* (**f**). *Vertical lines* indicate the moment at which 95% of final leaf length and final leaflet area are reached in well-watered (*solid line*) and water-stressed plants (*dashed line*) (**a**, **d**). Each value was the average of 12 plants per treatment. Bars indicate S. E

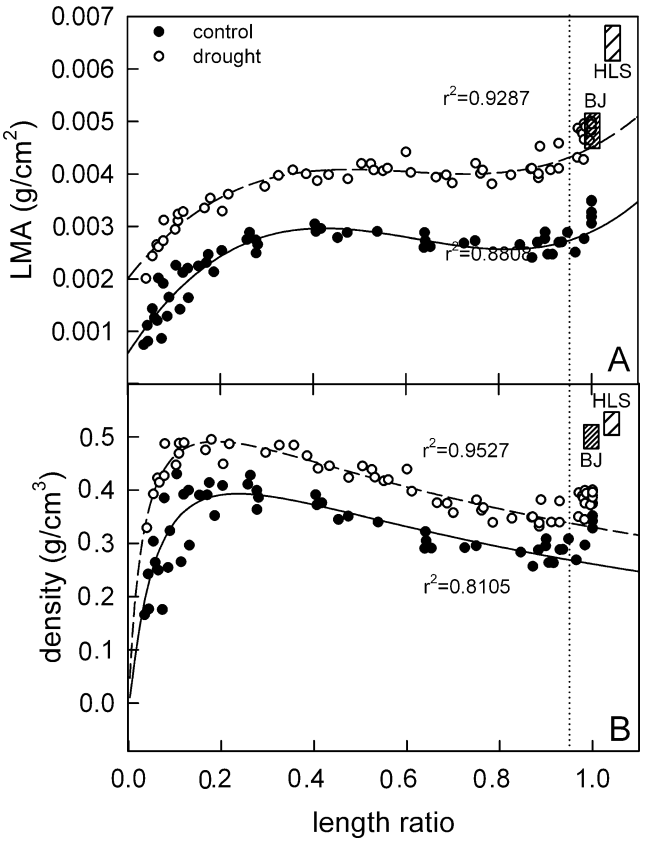

Fig. 2 Changes in *LMA* (**a**) and leaf density (**b**) during leaf development, expressed as leaf-length ratio. Length ratio is the ratio of growing leaflet area to final leaflet area. The vertical dotted line indicates 95% of final leaf length. Regression functions were fit for the data from 1% to 95% of final leaf length. r^2 is shown in the plot for each fit function. Field observations from mature trees in sites BJ (Beijing Forestry University) and HLS (Helan Mountain) are indicated by *vertical bars* for comparison (paper I of this series)

area did not differ significantly between treatments while at 50% of final leaf length, both epidermal and mesophyll cell number per cross area under water stress was significantly greater than controls ($P<0.01$). However, at 100% of final leaf length, only epidermal cell number was significantly reduced under water stress.

4 Discussion

There has been a growing interest in the factors influencing leaf morphology within the crown of large trees. Recent published reports have been appropriate in terms of scope and design for ecological studies, i.e., determining the range of variation among diverse species and using statistical approaches to find correlations that help one to hypothesize the underlying mechanisms that might account for the variation. In this regard, there have been some notable large-scale survey studies: (1) Sack et al. (2006) analyzed six deciduous species growing in an arboretum, (2) Cavaleri et al. (2010) surveyed a wide range of neo-tropical species of varying growth form (herbaceous, palms, epiphytes, trees, and lianas), (3) Oldham et al. (2010) focused on some of the largest known trees (*Sequioia sempervirens*). The "independent" factors that have been assessed include height, position in the canopy (inner versus outer), and irradiance, and these have been correlated statistically with one or more "dependent" variables such as leaf mass per unit area, some measure of leaf size (length, width, area, and/or thickness), or more cellular-scale measures (cuticles, stomates, mesophyll porosity, or cell size). The limitation of the statistical approaches used (maximum likelihood analysis or principle component analysis) relates to the conundrum that the independent variables identified might be interdependent through a third independent parameter and that a statistical analysis does little to address the underlying mechanisms that result in the plasticity of leaf morphology. We argue that both light and height combine to influence water potential which, in turn, controls growth and leaf morphology. For example, while Cavaleri et al. (2010) concluded that height is more important than light, Sack et al. (2006) concluded that light more than height determines leaf morphology. However, the latter study makes passing reference to a complicating factor: "The exposed leaves may also be 'stunted' by the irradiance and/or by the associated higher temperature and vapor pressure deficits, which might reduce leaf water status." This point deserves more attention.

Light (I=net irradiance) and height (h) might not be totally independent if a major independent factor influencing leaf morphology turns out to be water stress. A simple soil-plant-atmosphere continuum model can be used to explain the interrelatedness of height and light (Tyree 1999).

$$\Psi_w = \Psi_{soil} - \rho g h - E(I)/R_p, \quad (1)$$

where Ψ_w is the water potential of a growing leaf, Ψ_{soil} is the water potential of the soil when the leaf is growing, ρ is the density of water, g is the acceleration due to gravity, E is the evaporative flux density, and R_p is the hydraulic path resistance from the soil to any given developing leaf. Equation 1 might be usefully interpreted in terms of the Lockhart growth model (Lockhart 1965).

$$RGR = m(\Psi_p - Y) = m(\Psi_w + \Psi_\pi - Y), \quad (2)$$

where RGR is the relative growth rate, Ψ_p is the turgor pressure driving growth, Y is the yield point, and m is the cell wall extensibility. In terms of water potential, $\Psi_w = \Psi_p + \Psi_\pi$ where Ψ_π is the osmotic potential. Diurnally Ψ_π remains relatively constant between day and night in plant cells because 90% to 98% of the change in Ψ_p is attributed to change in Ψ_w. This follows because Ψ_π is inversely proportional to cell volume, and cell volume changes only a few percent diurnally. The only exception is when osmotic adjustments occur in some species in response to long-term water stress, where

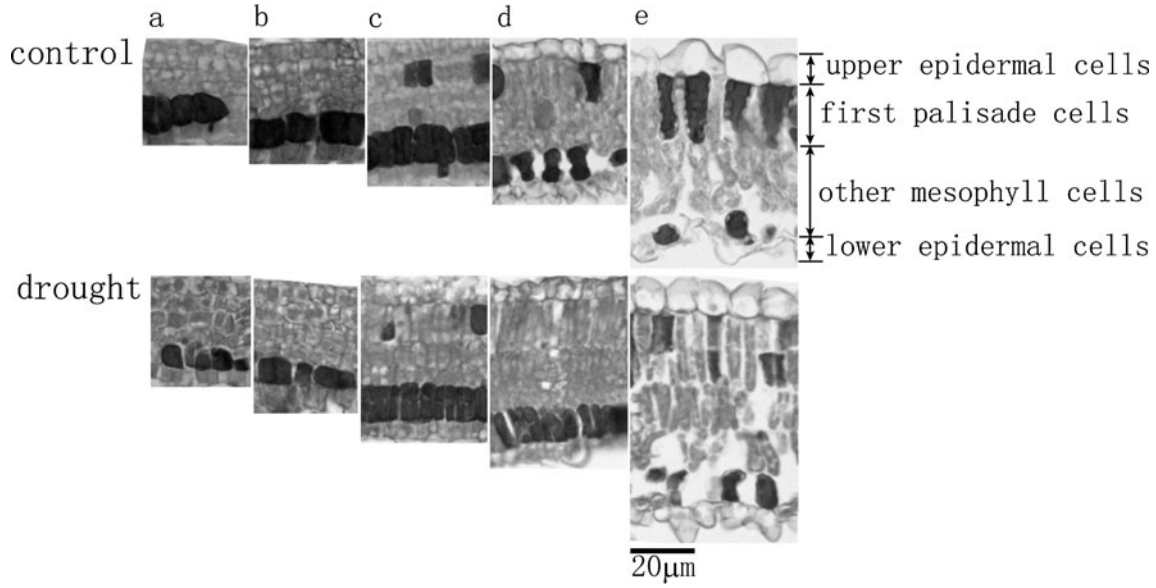

Fig. 3 Leaflet anatomy in control and drought treatments at five growth points: 5% (**a**), 10% (**b**), 25% (**c**), 50% (**d**) and 100% (**e**) of final leaf length. Cells were divided into four types from upper side of leaf to lower side: upper epidermal cells, first palisade mesophyll cells, other mesophyll cells, and lower epidermal cells

persistently low Ψ_w can induce a change in Ψ_π which is still thought be constant diurnally under persistent water stress.

Equation 2 describes only the impact of Ψ_w or Ψ_p on instantaneous growth rate whereas final leaf and cell size will depend on the integral of Eq. 2 over the entire period of growth which in the case of *Robinia* is 30 to 40 days in our study and typical of that in other woody species (Meinzer et al. 2008). While this paper does not parameterize *m* and *Y* in Eq. 2, this is the subject of the third paper in this series. But it is obvious from Eq. 1 that height and light co-contribute to Ψ_w.

Water stress (Ψ_w) is co-influenced by soil water stress, height, and *E*; *E* in turn is a function of *I*, which is the intended meaning of *E*(*I*) in Eq. 1. Evaporative flux rate depends on the energy needed to vaporize liquid water, and the majority of the energy is derived from net radiation. Hence, *E* is often a linear function of *I* (Tyree 1999). The

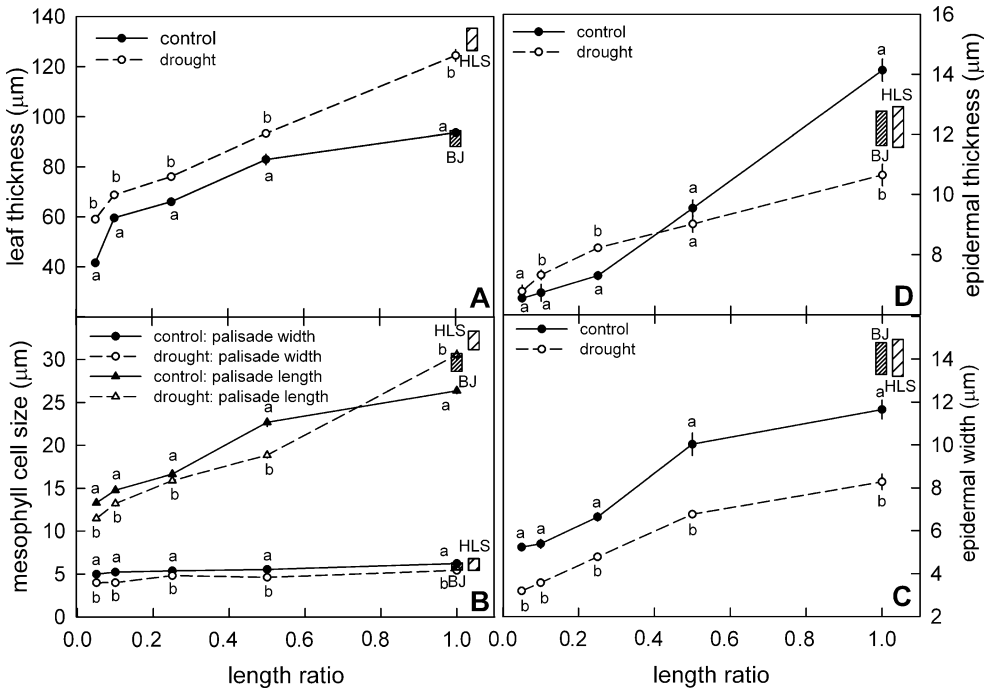

Fig. 4 Leaflet thickness (**a**), width and length of first-layer palisade mesophyll cells (**b**), width (**c**), and thickness (**d**) of upper epidermal cells at five growth points: 5%, 10%, 25%, 50%, and 100% of final leaf length. Each mean value at each growth point was the average of 12 trees. *Different letters above the error bars* indicate significant differences between treatments ($P<0.05$). *Bars* indicate SE. Field observations from mature trees in sites *BJ* (Beijing Forestry University) and *HLS* (Helan Mountain) are indicated by *vertical bars* for comparison (paper I of this series)

Table 2 Summary of upper epidermal and mesophyll cell number per cross area (number/mm^2) at five growth points during leaf development (5%, 10%, 25%, 50%, and 100% of final leaf length) in the two treatments

Length ratio	Epidermal cells			First-layer palisade cells			Other mesophyll cells		
	Control	Drought	*P* value	Control	Drought	*P* value	Control	Drought	*P* value
5%	6714±493a	4072±195b	<0.001	6008±601a	4172±41b	<0.001	24537±925a	16323±1299b	<0.001
10%	4195±227a	4367±171a	0.11	4299±192a	4327±208a	0.26	17796±1233a	18352±891a	0.093
25%	4137±124a	3721±160a	0.08	3937±86a	3614±110a	0.41	16756±707a	15344±569a	0.078
50%	2088±180a	2879±121b	<0.01	2752±121a	3171±93b	<0.01	10307±508a	12849±397b	<0.01
100%	1348±82a	784±46b	<0.01	1858±41a	1747±88a	0.091	5756±245a	5299±267a	0.082

Values are means ± SE. Different letters behind the values indicate significant differences between treatments ($P<0.05$)

contribution of E to Ψ_l is inversely proportional to the path length resistance (R_p) for water flow from the soil to the growing leaves. Also, leaves higher up in a canopy tend to have higher R_p values. So h and I are far from being independent from each other if we accept the hypothesis that growth rate is regulated by Ψ_w through some kind of Lockhart growth equation.

The present study was done to evaluate to what extent the morphological plasticity observed in adult Robinia leaves along tree height (paper I of this series) were attributable mainly to height-driven hydraulic constraints. In this experiment, the sole difference between treatments was a water potential difference comparable to that previously measured between the top and bottom of the crown of 20-m-tall Robinia trees growing in a temperate sub-humid site and in a temperate arid site (BJ and HLS sites, respectively, in paper I of this series). Our data showed that this imposed hydraulic constraint resulted in a significant variation in leaf morphological characteristics, such as smaller leaf area, higher *LMA*, and density (Figs. 1 and 2). These changes followed the same trend previously observed between leaves developed at the bottom versus the top of the canopy of adult trees (paper I of this series). Our data are in agreement with a study performed on *Quercus rubra*, which conclude that hydraulic limitations imposed by crown placement determine final size and shape of leaves Zwieniecki et al. (2004). This conclusion was bolstered by the assertion that irradiance, air temperature, and relative humidity were similar at the top and bottom of the crowns ($h<18$ m) throughout the period of leaf growth until leaf size reached more than 95% of final leaf size (Zwieniecki et al. 2004).

In addition to the main objective to assess the role of hydraulic limitations in the resulting leaf morpho-anatomical characteristics, our study also sought to gain a better understanding of the mechanisms underlying leaf morphological plasticity. One important mechanism to understand is the biomechanics of leaf growth and how this responds to water stress. In most previous studies, correlations were performed between final mature leaf morphology and Ψ_w, h, and I parameters without specific regard to documenting Ψ_w, h, and I when leaves were growing nor the tempo of growth. Our study is unique in that it addresses the tempo of growth, controls Ψ_w during leaf growth, and documents how the temporal growth process results in different morphologies. The mechanisms resulting in plasticity of leaf morphology can potentially involve both cell division and cell enlargement. Water stress affects leaf growth at the cellular level in either of two growth phases. During the first growth phase (before 25% of final leaf length), leaf growth is the consequence of coordinated cell division and tissue expansion (Pothig and Sussex 1985), which is reflected by low *GR* and high *RGR* (Fig. 1). Inhibition of cell division in younger leaves (i.e., before 5% of final leaf length) results in fewer epidermal and mesophyll cells per leaf under water stress (Table 2). During the second growth phase, leaf growth mainly depends on cell expansion (Granier and Tardieu 1998). We found that both palisade mesophyll and epidermal cell size were significantly reduced under water stress (Fig. 4). Smaller final leaf size under water stress is the consequence of lowered leaf growth rate despite prolonged growth duration in stressed versus control plants (Fig. 1). Increased leaf growth duration under water deficit is usually suggested to be related to cell division in the first growth phase (Aguirrezabal et al. 2006; Pereyra-Irujo et al. 2008). Reduced leaf growth is mainly attributed to smaller *GR*, which is closely associated with lower cell expansion during the second phase (Fig. 1; Pereyra-Irujo et al. 2008). Overall, our data suggest that water stress affects leaf morphology via its effect on both cell division and expansion.

The activity of cell division and expansion during leaf growth might be reflected from the temporal changes of *LMA* and density (Fig. 2). After leaf emergence, before leaf rapid expansion, mesophyll cell number increases (Table 2) and new cell wall buildup may result in increase of LMA and leaf density. During leaf rapid expansion, cell volume expansion (Fig. 4) leads to the decline of leaf density, while

secondary cell wall deposition at maturity might lead to a further increase in leaf density (Jurik 1986).

During leaf growth, *LMA* and density were significantly higher under water stress, which can be explained by the difference in cell number and cell size during leaf growth observed between treatments (Table 2; Fig. 4). At 5% of final leaf length, water stress inhibits cell division and in turn cell number per cross area was significantly smaller under water stress than controls. At 10% and 25% final length, total cell number per across area is not significantly different between treatments (Table 2), but leaf density and LMA in water-stressed plants were still higher than in controls. The one reason might be that cell wall properties are changed under water stress, which could be reflected by cell wall lower extensibility m and higher yield threshold Y in Eq. 2 (Matthews et al. 1984; paper III of this series). The other more likely reasons are explained in Zhang et al. (2011). Final leaf density and LMA values found in this study were smaller than those observed in the field (paper I of this series). Two possible reasons may account for these differences: (1) field trees experience five times higher maximum light intensities than in our controlled environment experiments, and it is known that leaves respond to high light by growing thicker mesophyll tissue (more layers of palisade mesophyll cells) to enhance light absorption (Hanson 1917), (2) mature leaves of seedlings may differ from leaves found on adult trees (Piel et al. 2002).

In conclusion, this study suggests that water stress can influence leaf morphology significantly via its effect on the leaf growth process, and the magnitude of the changes are comparable to what we observed in mature *Robinia* trees (vertical bars in Figs. 2 and 4). Although the Ψ_w values in this study differ quantitatively from the field study on *Robinia* (paper I of this series), the differences were likely due to factors in Eq. 1 and that measurements were made in summer on mature leaves in the field. The lower mid-summer values of Ψ_w were likely because of lower Ψ_{soil} higher E and higher R_p than the values in spring. The temporal changes of leaf and cell properties during leaf growth were different between the two treatments. The impact of water stress on growth parameters in the Lockhart growth equation (Lockhart 1965) will be addressed in the series paper III.

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