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Hydraulic limitations imposed by crown placement determine final size and shape of *Quercus rubra* L. leaves

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ABSTRACT

The canopies of large broad-leaf trees exhibit significant heterogeneity in both micro-environmental conditions and leaf morphology. Whether the visible differences in the size and shape of leaves from the top and bottom of the crown are determined prior to bud break or result from different patterns of leaf expansion is not known. Analysis of ontogenetic changes of both the degree of lobing and vein density in *Quercus rubra* demonstrates that leaves throughout the crown are identical in size and shape at the time of bud break. Morphological adaptation to the local micro-environment takes place during the expansion phase and starts after the determination of the vascular architecture has been completed. Leaves from the bottom of the crown undergo greater expansion in the tissue close to the main veins than occurs either in the more peripheral tissue of the same leaf or anywhere in leaves from the top of the crown. This results in a water transport system that is well suited to the low evaporative rates near the bottom of the crown, but inadequate for the conditions found at the top of the tree. Acclimation of leaf form and function based upon differential expansion may be entirely driven by the local hydraulic demand during the expansion phase, resulting in leaf size and vein density being determined during development by the same hydraulic properties which will constrain the size of leaf that can be functionally supported at maturity.

Key-words: Leaf shape; leaf lobing; leaf expansion; leaf development; *Quercus rubra*; leaf hydraulic properties.

INTRODUCTION

Morphological attributes of leaves are known to vary substantially within individual plants and this phenotypic plasticity is typically thought to be related to environmental variability (Niinemets, Kull & Tenhunen 1999; Koike *et al.* 2001a; Hanba, Kogami & Terashima 2002). The earliest stages of leaf development, including primordium formation, most cell divisions, and overall tissue patterning, occur while the nascent leaf is largely sheltered from the environment. Prior to bud break, direct genetic and hormonal control should be the primary influences upon development (Chan *et al.* 1998; Wyrzykowska *et al.* 2002). However, as development proceeds through cell expansion and differentiation, the growing leaf is increasingly exposed to the surrounding environment and there is increasing opportunity for phenotypic divergence. What is not clear is the relative extent to which morphological variability is the result of the induction of alternative genetic pathways in response to environmental cues (e.g. day length, light quality) or instead is the result of a more direct response to environmental limitations imposed upon the growth process.

In discussions of morphological variability, leaves are often divided into two groups based on their light environment: sun and shade (Feild *et al.* 2001; Guignard, Boka & Barbacka 2001). Light is often considered to have a major influence on leaf development and a common research goal has been to understand the signalling processes that link light conditions with photosynthetic pigment production, cuticle thickness, sclerification, vein and stomata density, and overall leaf size, shape, and thickness (Poole *et al.* 1996; Onwueme & Johnston 2000; Koike *et al.* 2001b; Terashima, Miyazawa & Hanba 2001). Light quality and quantity are undeniably an important part of the story and may be the primary determinant for some of these morphological attributes. However, the distinction between sun and shade leaves is actually a conflation of several environmental variables and does not simply reflect a gradient in light availability. In particular, the division between sun and shade leaves also reflects a gradient in the demand for water.

The ability of a leaf to carry out its role as a photosynthetic organ requires that its demand for water be balanced with the capacity of the vascular system. Because rates of water loss from leaves, to a first approximation, scale linearly with leaf area, leaf size will be constrained by the limits set by the cavitation thresholds within the vascular system (Nardini & Salleo 2000; Nardini, Tyree & Salleo 2001). In addition, leaf size will be constrained by the need to distribute water in a manner that allows the periodic opening of stomata for carbon dioxide uptake to occur in all parts of the lamina (Zwieniecki *et al*. 2002). These limitations upon the maximum rate of water transport and requirements for equitable water distribution must be followed in all leaves despite the existence of a broad range of leaf morphologies and micro-environments within the crown of an individual plant. Variation in leaf size and shape as well as in evapo-

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rative demand could be accommodated by the production of additional veins when necessary, although the limitations of the maximum water flow into the leaf through the petiole would still apply. Alternatively, efficient leaf function could be accomplished by maintaining the same vein system regardless of micro-environment and then using environmental cues to dictate the extent to which photosynthetic area increases during leaf expansion. By this mechanism, vein density and leaf size and shape would ultimately be determined by cell expansion, a process dependent upon water uptake by the cell vacuole. Leaf size, shape, and vein density could therefore be directly controlled by the same hydraulic limitations that will eventually govern leaf function. In this paper we investigate the morphologies of *Quercus rubra* leaves through ontogeny in relation to hydraulic properties and location within the tree crown to determine the role that hydraulics play in leaf development.

METHODS

Plant material

Leaf material was collected from several trees of *Quercus rubra* L. growing in the Arnold Arboretum during spring 2001. An aerial lift was used to collect leaves from the outer part of the top (approximately 15–18 m above the ground) and bottom (5–7 m above the ground) of the tree crown. Sampling started several days before bud break and continued until leaf expansion was completed. During each collection time, 11 leaves from each canopy height were randomly selected from several branches and flat dried for shape and size analysis. In addition five leaves or buds were preserved in formalin : acetic acid : ethyl alcohol (FAA; 1 : 1 : 9) for microscopic investigation.

Micro-environmental conditions within the crown were monitored throughout leaf expansion in 2003 using Hobo dataloggers (Onset Computer Corporation, Bourne, MA, USA). Air temperature, relative humidity and irradiance were recorded for two locations (top and bottom of crown) at 10 min intervals. Irradiance was later converted to photosynthetically active irradiance using 0.45 conversion factor (Nobel 1983).

Size and shape analysis

Images of carefully flattened leaves were acquired with a high-resolution scanner and used to determine leaf area and the area of the smallest convex polygon enclosing the leaf lamina (hereafter, the leaf envelope). A 'lobe index', calculated as the ratio of leaf area and leaf envelope area ($cm^2\ cm^{-2}$), was introduced to compare leaf shapes. This index is a size independent description of the filling of space by the leaf lamina. A lobe index of 1 indicates an entire leaf (no lobing), whereas values approaching 0 indicate an increasing proportion of the leaf envelope area being occupied by the spaces between lobes. Image analysis was performed using the IMAGEJ (National Institute of Health, Bethesda, MD, USA) freeware program.

Relative growth rate was calculated from measurements of leaf area according to the formula $(\log_{10}A_2 - \log_{10}A_1)/t$, where A_x is the average leaf area at time x and t is the elapsed time (days). Standard errors were calculated according to Beers (1957).

Transpiration rate

Transpiration was measured at the end of July 2001 at the same two crown heights from which leaves were collected for the expansion growth analysis. Transpiration rate was measured around 1000 h using a portable porometer LiCor1600 (Li-Cor Inc., Lincoln, NE, USA) for five leaves at each height.

Vein density analysis

Vein density was determined for leaves collected 10 and 22 d after bud break, the latter being near completion of leaf expansion. Three leaves from each height were used in the analysis. On each leaf, six locations were selected for measurements of vein density: two sites at the cusp of the sinus between the third and fourth lobe (one marginal and one near the midvein) and four sites in the fourth lobe, including two half-way along the lobe (one marginal and one near the second-order vein) and two marginal areas at or near the distal end of the lobe (see Fig. 3c). Regions of interest were adhered to microscope slides and photographed using a light microscope. Prior storage of leaf material in FAA resulted in the loss of most pigments from the leaf, making it possible to see all of the fine vein network without further clearing or staining. Vein density was determined using the software package IMAGEJ. We used nested analysis of variance to test for main effect significance, where position on the leaf was nested within the location of the leaf on the tree. A Bonferroni analysis was used for *post-hoc* analysis of the significant differences among each leaf position and crown location (Sokal & Rohlf 1981).

Pressure gradient measurements

The method for determining spatial patterns of pressure dissipation across the leaf venation followed that described in a previous study (Zwieniecki *et al*. 2002). The basic approach consisted of supplying water at a known delivery pressure and flow rate to the petiole of an excised leaf and then measuring the pressure drop to individual veins around the leaf. Pressure dissipation in a transpiring leaf can then be estimated by scaling each measurement to the same transpiration rate and combining measurements made on different leaves to construct a spatial map of pressure dissipation across the leaf lamina.

The actual measurement procedure consisted of cutting a leaf from the branch and attaching the petiole to the outflow of a laboratory-built flow meter using a compression fitting (Zwieniecki *et al.* 2000). Water was then supplied to the petiole at a low pressure (approximately

0.02 MPa), allowing a small amount of water to flow into the leaf. The leaf was viewed with a dissecting microscope and the desired vein was microsurgically severed and attached to a pressure probe (Zwieniecki *et al*. 2002). In this study we present only results from the highest order veins. The microcapillary tip and all cut surfaces were then sealed with cyanoacrylate glue. Special attention was paid to seal all open leaf surfaces and small veins to avoid any pressure drop due to leaks caused by injury. During pressure measurements the leaf was inspected for leakage under the microscope and if any leak was observed the measurement was discarded. The pressure in the microcapillary was recorded after the 30 to 60 min needed for flow and pressure to stabilize. Upon completion of the experiment the petiole was cut close to the compression fitting and its cut surface plugged with glue to test for leaks around the seal. In no case was a leak detected. A total of 21 and 19 leaves from the bottom and top of the canopy, respectively, were used in this analysis. One to three measurements were performed on each leaf for a total of 51 and 53 pressure determinations. Measurements of pressure dissipation were recalculated for a common flow rate. Pressure dissipation maps were constructed using the Delaunay triangulation method (MATLAB 6.0, The MathWorks Inc., Natick, MA, USA) followed by the triangular surface plot technique (MATLAB 6.0, The MathWorks Inc.).

Thermographs of the leaf surface

Infrared images of transpiring leaves were used to obtain an estimate of the spatial distribution of transpiration from leaves obtained from both the top and the bottom of the canopy when exposed to the evaporative conditions of both canopy locations. Due to logistical constraints, these measurements were made on *Q. rubra* trees growing at the Harvard Forest, Petersham, MA, USA. Thermal sensitivity of the infrared camera (ThermaCAM PM 695; FLIR, Danderyd, Sweden) was 0.08 °C at 30 °C. Prior to making these measurements, several leaves were collected and their emissivity determined indoors using black electrical tape as a reference point (emissivity of 0.98). Average leaf emissivity was 0.79 and this value was used to determine leaf temperature in the field. Leaves used in analysing the temperature profile across the leaf lamina as a function of canopy position were collected around noon. Two branches (approximately 0.5 m long) were cut from the tree: one from the top of the crown, 15 m above the ground, and one from the bottom of the crown, 5 m above the ground. Branches were cut under water and the cut ends were kept in water at all times during later manipulations. Two leaves, one from each branch (while remaining attached to the branch) were placed side-by-side in a wooden frame. Transparent nylon string was used to keep the leaves flat and at the same orientation. The wooden frame was then raised to the elevation of the top of the crown and exposed to full sun and ambient wind at that level (i.e. high evaporative demand conditions). After approximately 30 min, a series of pictures were taken. The entire system was then moved down to the environment of the leaves growing at the bottom of the crown and a second series of pictures was taken after another 30 min allowed the leaves to adapt to the new conditions.

RESULTS

Photosynthetically active irradiance, air temperature, and relative humidity were similar at the top and bottom of the crown at the onset of leaf expansion (Fig. 1). Temperature and relative humidity remained similar at the two heights throughout the period of leaf growth; however, beginning approximately 27 d after bud break, cumulative photosynthetically active irradiance (MJ m^{-2}) measured at the top of the canopy indicated higher levels of incident radiation than that reaching leaves at the bottom of the crown. This change in irradiance coincides with increased shading of the lower crown as the leaves expand.

Maximum relative growth rates were higher in leaves at the bottom of the crown, compared with leaves from the top of the crown, although the overall pattern was similar (Fig. 2). Leaf expansion of lower canopy leaves began several days prior to bud break in the upper crown, however, maximum rates of expansion occurred at about 10 d post bud break in both groups of leaves. Leaf expansion took place during a nearly rainless period in 2001, however, a small precipitation event near the end of leaf expansion resulted in a small increase in relative expansion rate.

The area of leaves from the top and bottom of the crown were indistinguishable up to the eighth day after bud break, after which time leaves from the bottom of the crown expanded more rapidly and were significantly larger (Fig. 3). The lobe index initially was approximately 0.5 (i.e. leaf lamina filled around 50% of the polygon outlining the leaf) for leaves from both crown locations and remained similar for the first 8 d after bud break. However, the lobe index gradually diverged during the maximum expansion phase, leading to a significant difference between leaves from the two crown heights by the end of leaf expansion. Leaves from higher in the crown maintained a lobe index of approximately 0.5, but leaves from the lower height changed from 0.5 to 0.7, reflecting a greater degree of space filling with wider lobes and less deeply incised sinuses (Fig. 3).

Early on (tenth day after bud break) there were no significant differences in vein density associated with crown height or position within the lamina (Table 1; Fig. 4). In mature leaves (end of the expansion phase), the average vein density was higher in the leaves from the top of the canopy than from the bottom ($P < 0.001$; Table 2). There was a significant change in vein density across the blade of mature leaves ($P < 0.001$). In leaves from both crown heights, vein density was greater near the margin and at the lobe tips (locations 5 and 6) and lowest in the proximity of the main vein (locations 1–3), although this gradient was much less pronounced in leaves from the top of the canopy where differences in vein density were not statistically significant (Fig. 4).

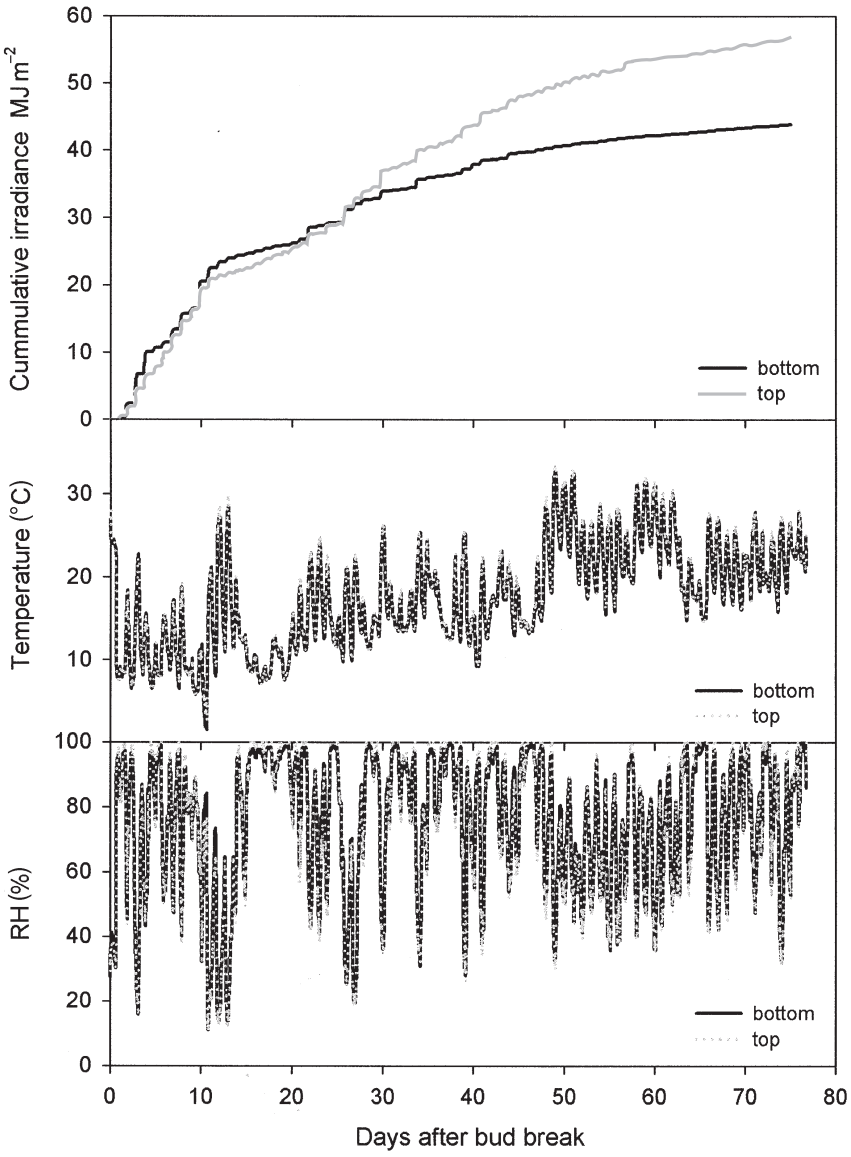

Figure 1. Micro-environmental parameters throughout the period of leaf expansion at the top (dashed line) and bottom (solid line) of a *Q. rubra* crown.

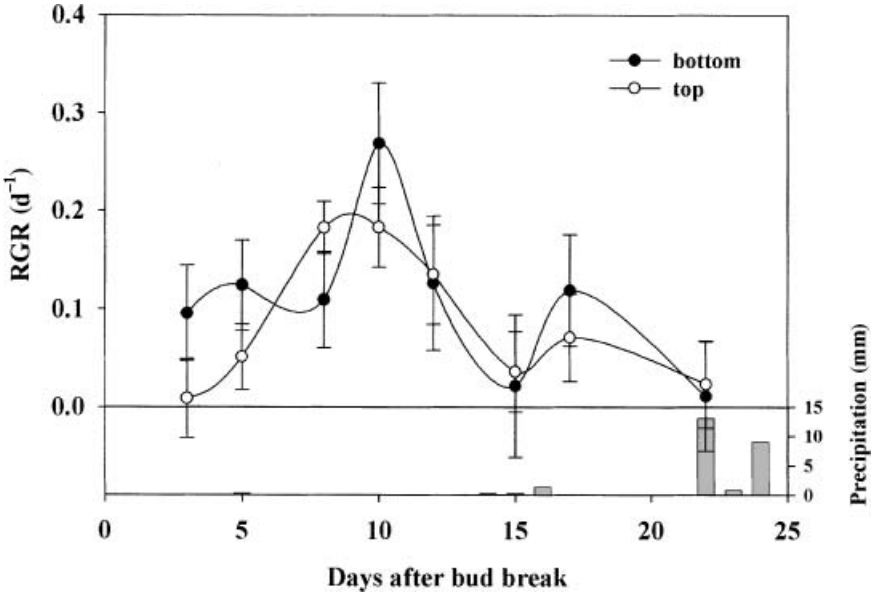

Figure 2. Relative growth rate (d^{-1}) of leaves from the top and bottom of the crown during leaf expansion. Vertical bars indicate the standard error of the mean. Lower panel shows precipitation events during the same period.

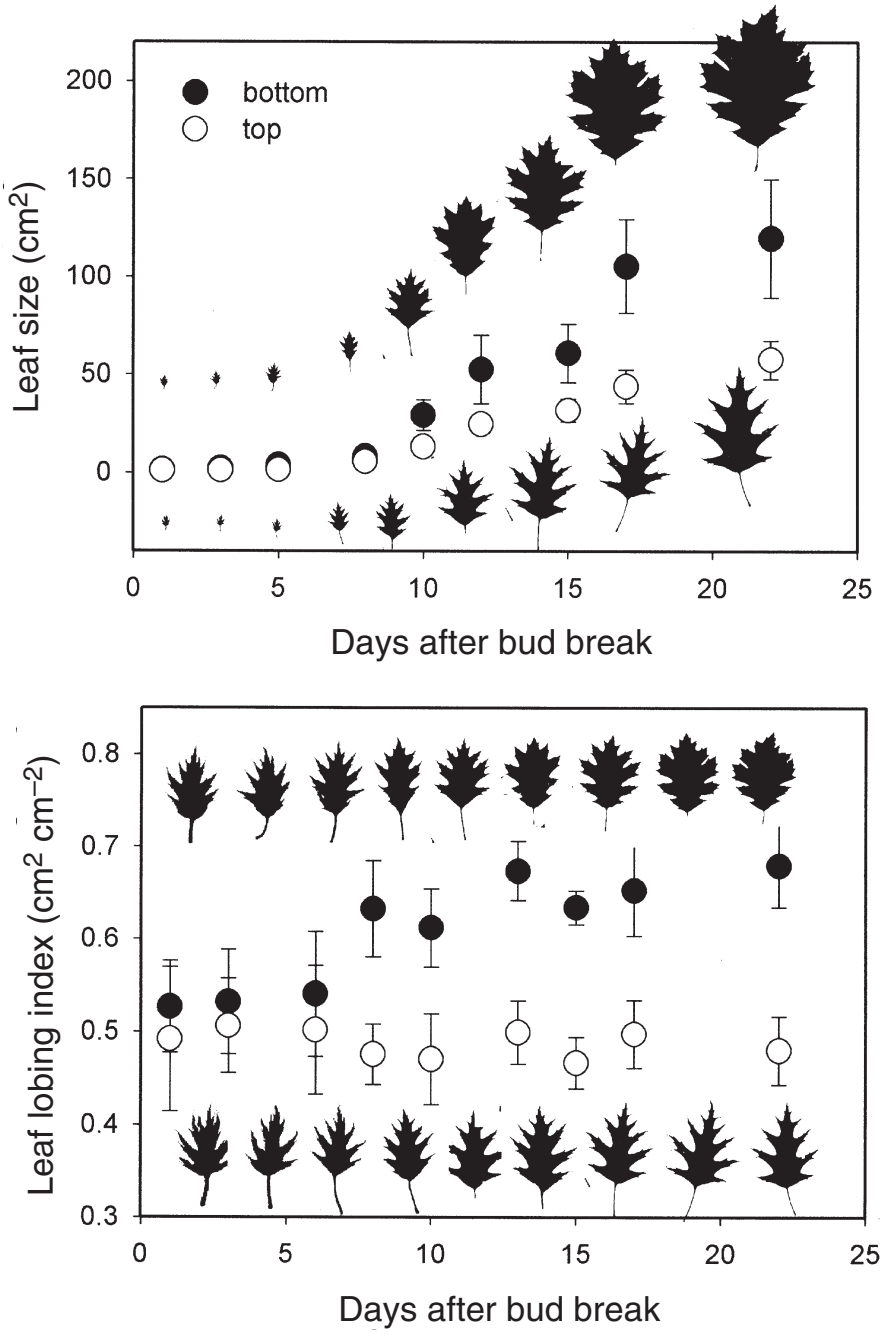

Figure 3. Changes in leaf size (top panel) and shape (lower panel) during leaf expansion for leaves growing at the top and the bottom of the canopy. The leaf lobing index was calculated as the ratio of the actual leaf area to the area of the smallest convex polygon enclosing the leaf lamina.

Table 1. Nested analysis of variance for vein density of leaves at one-fifth of the final size

	SS	d.f.	MS	*F*	*P*
Intercept	12578.13	1	12578.13	5511.1	0.000
Location within the tree	3.02	1	3.02	1.3	0.262
Position on the leaf	25.61	10	2.56	1.1	0.386
Error	54.78	24	2.28		

SS, sum of squares; d.f., degrees of freedom; MS, mean square.

The hydraulic design of each of the two mature leaf morphological types is well suited for the demands of its micro-environment. The transpiration of leaves from the top of the crown averaged 7.8 mmol m^{-2} s^{-1} at 1000 h on a sunny warm midsummer day. At that rate of transpiration these leaves would experience a drop in pressure across the leaf blade of only a few tenths of a MPa (Fig. 5A). At the transpiration rate measured for the leaves from the bottom of the crown (2.8 mmol m^{-2} s^{-1}), the pressure drop across the leaf blade would be similar. However, if leaves from the bottom of the canopy had transpiration rates similar to those at the top of the canopy, the estimated pressure drop across the leaf blade would be over 1.0 MPa and surpass

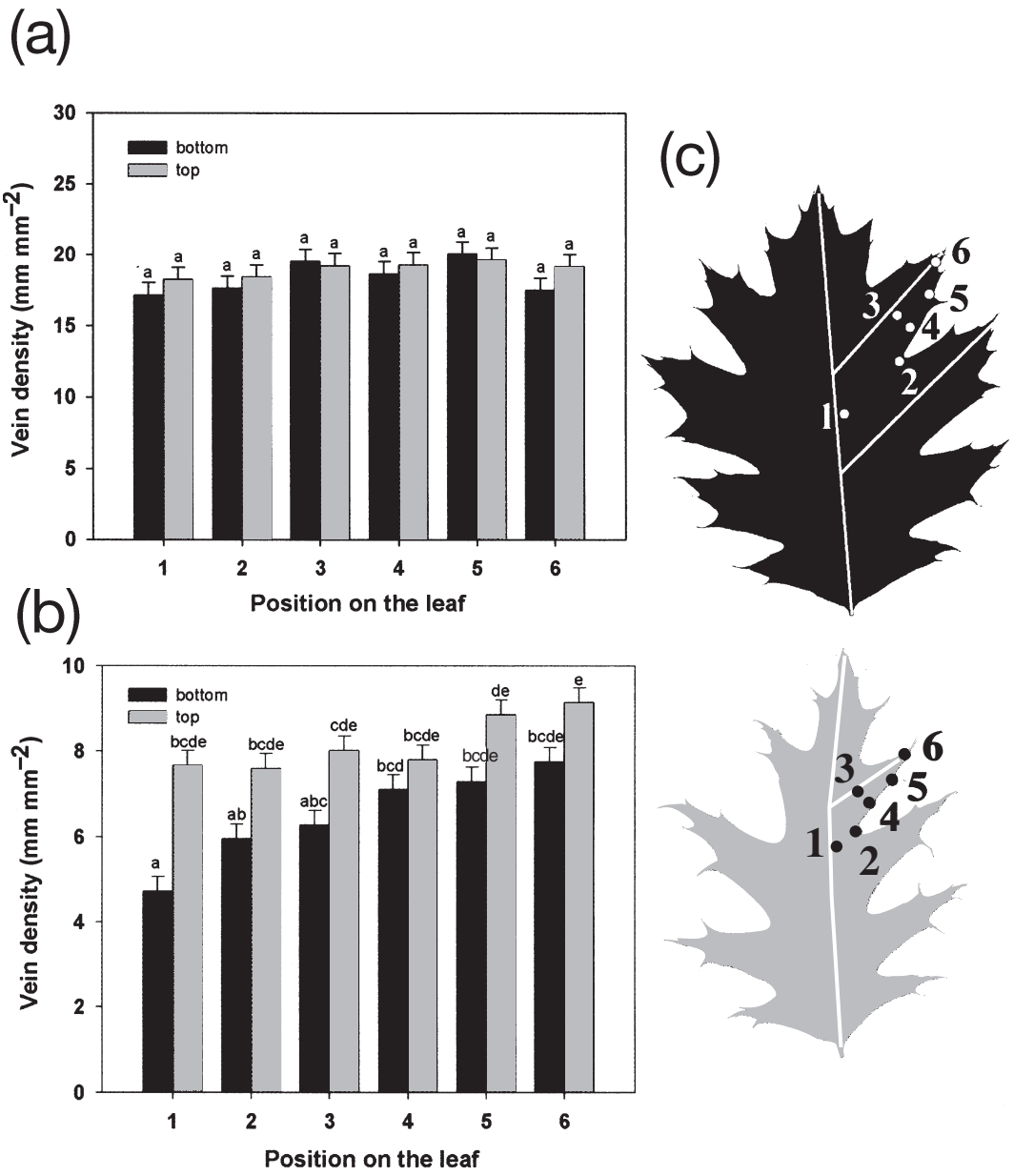

Figure 4. Vein density as a function of lamina position for leaves growing at the top and bottom of the canopy. (a) vein density of leaves collected 10 d after bud break. (b) vein density of fully expanded leaves. (c) diagram showing sample locations.

1.5 MPa in the lobes. Under such conditions, stomata could not open. If leaves from the top of the canopy were limited to the transpiration rates found at the bottom of the canopy, the estimated pressure drop across the leaf would be less than 0.2 MPa. Under these conditions, photosynthesis could occur, but such a low pressure drop suggests an inefficient use of resources; the investment in vasculature would be more than is necessary. Note that although boundary layer effects have not been taken into account, they would only accentuate the differences in transpiration rate between crown heights.

The above analysis is supported by comparison of the temperature profiles of leaves of each morphological type subjected to the conditions of high and low evaporative demand found at the top and bottom of the canopy (Fig. 5B). When exposed to the high evaporative demand at the top of the crown, leaves originally collected from the top of the crown were slightly warmer along the midvein than in peripheral tissues (reflecting the higher capacity for convective cooling of the lobes), but the relatively low temperatures and weak overall gradient suggests that each leaf was able to transpire across its entire surface. However, leaves from the bottom of the crown exposed to high evaporative demand were much warmer at the periphery than near the midvein. This suggests that the transport capacity was insufficient to meet the evaporative demands resulting in stomatal closure. When leaves were exposed to low evaporative demand at the bottom of the crown, both types of

leaves were cooler then in the previous experiment and both presented temperature profiles that were relatively uniform and similar in absolute temperature. This suggests that both leaf types were able to freely transpire across their entire surface.

DISCUSSION

During ontogeny, the adjustment of growth parameters results in a leaf morphology suitable for its micro-environment. Some environmental parameters may affect development at the time of leaf primordia and bud formation, the year before bud break and leaf maturation. However, the environmental conditions during bud formation may not be a sufficient predictor of the eventual environment of the mature leaf. Many morphological features of the leaf must therefore be shaped during the later stages of leaf development. Early after bud break, every leaf on a tree may well experience 'sun' leaf irradiance. However, growth of the leaves themselves leads to changes in micro-environmental parameters within the crown such as irradiance, humidity, and wind speed. Thus, the micro-environmental conditions that will be experienced by the mature leaf cannot be reliably predicted at the time of bud break. Furthermore, the micro-environment of the mature leaf cannot be predicted at the time of bud formation during the previous season due to stochastic processes such as branch dieback, activity of pests, and over-shading by competitors. Such unpredictability requires that leaf expansion continuously respond to current environmental inputs. As our data shows, both the initial vein density and lobe index of newly emerging leaves was similar for both heights. However, when expansion growth was at its maximum, leaves from the bottom of the crown gradually filled more space between the lobes, whereas the shape of leaves at the top did not change during expansion.

Measurements of vein density demonstrate that leaf expansion resulted in a significant and non-uniform decrease in leaf vein density. Vein density depends on the size of the areoles in relation to their perimeter. If leaf

Table 2. Nested analysis of variance for vein density of fully expanded leaves

	SS	d.f.	MS	*F*	*P*
Intercept	1944.91	1	1944.91	5490.9	0.000
Location within tree	25.01	1	25.01	70.6	0.000
Position on leaf	24.76	10	2.48	6.9	0.000
Error	8.50	24	0.35		

SS, sum of squares; d.f., degrees of freedom; MS, mean square.

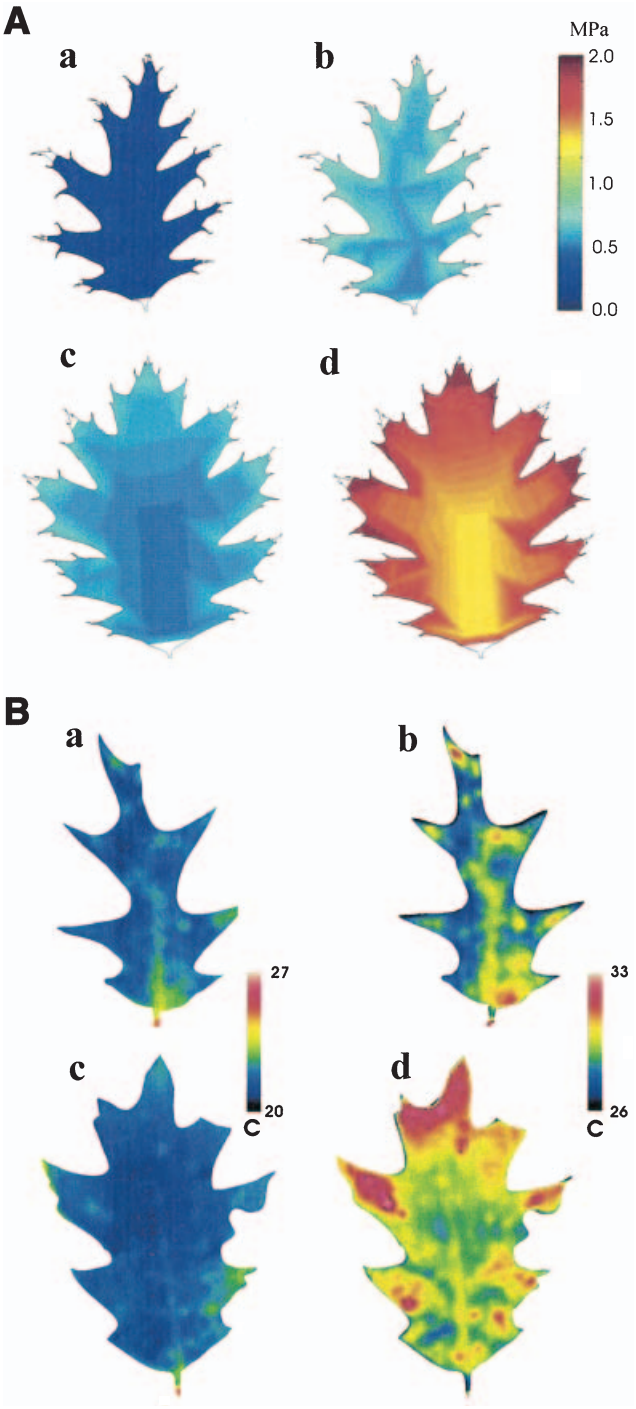

Figure 5. (A) Estimated pressure drops in the highest order of veins for leaves growing at the top and bottom of the crown. (a) Pressure drop in leaves from the top of the crown experiencing the transpiration rates found at the bottom of the crown (2.8 mmol m^{-2} s^{-1}). (b) and (c) Pressure drops expected for leaves from the top (b) and bottom (c) of the canopy under the maximum transpiration rates of their natural canopy placement. (d) Pressure drop in the veins of leaves from the bottom of the canopy experiencing the evaporative demand of leaves from the top of the canopy (7.8 mmol m^{-2} s^{-1}). (B) Thermographs of the leaves collected from the top and bottom of the crown. Images on the left, (a) and (c), are of leaves supplied with water and placed at the bottom of the crown. Images on the right side, (b) and (d), are of leaves supplied with water and allowed to transpire at the top of the crown. Emissivity of the leaves was measured as 0.79. Ambient air temperature was 24 °C. Note the change of temperature scale for the two sets of images corresponding to the two evaporative environments.

expansion is uniform, vein density should decrease by a scaling factor that is the inverse of the square root of the area scaling factor. If new veins are formed during expansion, then the scaling factor for vein density will be larger. Leaf area increased by a factor of 4.08 for the bottom of the canopy and 4.35 for the top between the two times at which vein density was measured. Thus, if expansion occurred uniformly in these leaves, vein densities would decrease by a factor of 0.49 and 0.48 for canopy bottom and top, respectively. However, the actual drop in vein density for leaves from the bottom of the canopy was by a factor of 0.27 near the main veins and 0.44 near the leaf margin. This indicates that overall leaf expansion exceeded the longitudinal growth of the veins (i.e. expansion led not just to an increase in areole size, but also a distortion of areole shape). Furthermore, the more pronounced drop near the main veins suggests that expansion of the leaf lamina was not uniform and that areas around the main and second-order veins undergo more expansion than areas along the leaf perimeter. This differential expansion accounts for the increase in lobe index. In leaves from the top of the canopy, vein density dropped by a factor of 0.41 near the main veins and 0.51 at the leaf margin, values which are both much more similar to each other and to the value that would be expected from uniform leaf expansion. This is reflected in the relatively constant lobe index during the growth of leaves from the top of the canopy.

The patterns of pressure dissipation across the vein network will depend upon both evaporative demand and the leakiness of the veins (Zwieniecki *et al.* 2002). The hydraulic capacity of a leaf vein system could be described as neither under- nor over-built if the leaf experiences a modest pressure drop of only a few tenths of a MPa across the leaf blade from petiole to fourth-order veins when transpiration is at midday levels. The predicted pressure dissipations and corresponding thermal images demonstrate that leaves of the morphology found at the bottom of the canopy would have insufficient hydraulic capacity to deal with the harsh conditions found at the top of the canopy, whereas leaves of the morphology found at the top of the canopy would be over-built for the lower evaporative demand at the bottom of the canopy. This is consistent with recent results that found higher leaf hydraulic conductance (kg m^{-2} s^{-1} MPa^{-1}) in sun versus shade leaves of four woody temperate species (Sack *et al.* 2003).

Adaptation to local evaporative demand starts relatively early but presumably after completion of cell division. During the early developmental stages, leaves are well protected from the environment by bud scales and later by trichomes. The lobe indices of the two leaf types start to diverge by the eighth day after bud break when the leaves are less then 20% of their final size. The complete vein pattern has also been determined by this point (i.e. no new veins are inserted), although their continued expansion requires that only the protoxylem is mature. This complete but only partially functional hydraulic distribution system must deliver the water required for expansion and cuticular transpiration. It is well known that water deficit limits leaf expansion (Frensch 1997; Munns *et al.* 2000). We suggest that limitations on the ability of the water delivery system to meet local demands across the leaf lamina play a major functional role in determining the extent of local expansion, thereby matching the size and shape of the mature leaf to its micro-environment.

Rather than anticipating future environmental conditions by the induction of complex developmental cascades in response to early environmental cues, our data suggest that leaves simply expand to the extent that their micro-environment allows. If the fine tuning of leaf shape is driven by changes in gene expression in response to local water demand, it would require cells to measure their degree of water stress. A simple alternative is that expansion will decrease if local cell pressure becomes too negative due to the pressure drop across the vein system. In this way, environmental cues translate directly into growth responses. This mechanism is elegant because leaf size and vein density are determined directly during development by the same hydraulic properties that will also constrain the size of leaf which can be functionally supported at maturity.

The effects of this relationship between micro-environment and expansion rate upon final leaf morphology have important implications for paleobotanical research. The expectation of differing gradients of vein density across leaves from the top and bottom of the canopy can be used to assess the likelihood that two distinct fossil morphospecies actually represent the sun and shade leaves of the same plant. This is particularly well suited for application to the fossil record because vein density is the most universally available of the traditional 'sun/shade' characteristics in leaf compression fossils (i.e. more so than palisade or cuticle thickness, stomatal density, etc.) and because variation in vein density is the only one of these characteristic than can be assessed within a leaf rather than between leaves. The expectation that canopy versus understorey leaves would have different patterns of vein density across the lamina may also allow the gathering of information concerning canopy structure and whole plant architecture from disarticulated leaves of unknown phylogenetic placement or plant habit.

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