Research paper

Patchy stomatal behavior during midday depression of leaf CO_2 exchange in tropical trees

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We investigated effects of heterogeneous stomatal behavior on diurnal patterns of leaf gas exchange in 10 tree species. Observations were made in middle and upper canopy layers of potted tropical rainforest trees in a nursery at the Forest Research Institute Malaysia. Measurements were taken from 29 January to 3 February 2010. We measured in situ diurnal changes in net photosynthetic rate and stomatal conductance in three leaves of each species under natural light. In both top-canopy and sub-canopy species, midday depression of net assimilation rate occurred in late morning. Numerical analysis showed that patchy bimodal stomatal behavior occurred only during midday depression, suggesting that the distribution pattern of stomatal apertures (either uniform or non-uniform stomatal behavior) varies flexibly within single days. Direct observation of stomatal aperture using Suzuki's Universal Micro-Printing (SUMP) method demonstrated midday patchy stomatal closure that fits a bimodal pattern in *Shorea leprosula* Miq., *Shorea macrantha* Brandis. and *Dipterocarpus tempehes* V.Sl. Inhibition of net assimilation rate and stomatal conductance appears to be a response to changes in vapor pressure deficit (VPD). Variable stomatal closure with increasing VPD is a mechanism used by a range of species to prevent excess water loss from leaves through evapotranspiration (viz., inhibition of midday leaf gas exchange). Bimodal stomatal closure may occur among adjacent stomata within a single patch, rather than among patches on a single leaf. Our results suggest the occurrence of patches at several scales within single leaves. Further analysis should consider variable spatial scales in heterogeneous stomatal behavior between and within patches and within single leaves.

Introduction

Plants regulate the exchange of CO_2 and water vapor with the atmosphere by adjusting their photosynthetic capacity and changing the apertures of stomata on the epidermis of their leaves. To prevent water loss and facilitate CO_2 diffusion to mesophyll cells, the stomatal aperture mechanism responds variably to environmental factors including light intensity, water status, temperature and nutrient supply.

Responses to environmental stimuli usually differ between adaxial and abaxial stomata on amphistomatous leaves (e.g.,

Travis and Mansfield 1981, Wang et al. 1998). Moreover, heterogeneous or 'patchy' stomatal behavior occurs on the surface of single leaves in response to water stress (Sharkey and Seemann 1989, Gunasekera and Berkowitz 1992), low humidity (Loreto and Sharkey 1990, Beyschlag et al. 1992, Mott et al. 1993) and exogenous abscisic acid (ABA) application (Downton et al. 1988, Terashima et al. 1988). This phenomenon, as well as non-uniform photosynthesis in response to environmental stimuli, has been observed in plants with heterobaric leaves, in which vertical extensions of bundle sheath

cells delimit the mesophyll and restrict the diffusion of CO_2 (Mott and Buckley 1998, 2000, West et al. 2005), whereas uniform stomatal behavior occurs in homobaric leaves that lack these vertical extensions (Terashima 1992). Concerning the differences in growth environment between the two leaf types, Wylie (1952) reported that plants with heterobaric leaves were found in temperate and dry areas, whereas those with homobaric leaves were found in warmer and wet regions. Kenzo et al. (2007) postulated that bundle sheath extensions might give heterobaric leaves an advantage over homobaric leaves in the severe stress canopy environment; this argument is based on the occurrence of different leaf types across the steep vertical microenvironmental gradient from forest floor to tree top. The bundle sheath extensions in heterobaric leaves may respond quickly to drought signals by, for example, reducing mesophyll water potential or increasing concentrations of ABA transported via the transpiration stream in bundle sheath extensions (Terashima 1992).

Heterogeneous stomatal behavior can be seen in plants under natural ecosystems during midday depression of photosynthesis (Beyschlag and Pfanz 1990, Beyschlag et al. 1992, Takanashi et al. 2006). The existence of this phenomenon under natural conditions is important because algorithms commonly used to calculate leaf conductance from water vapor exchange measurements assume homogeneously open stomata (see von Caemmerer and Farquhar 1981). If there is stomatal patchiness, then it results in error in the calculations, and the effects of stomatal behavior on midday depression of photosynthesis are underestimated. A few studies determined the effects of patchy stomatal behavior on gas exchange in the field. Using a pressure-infiltration method and numerical analysis, Takanashi et al. (2006) demonstrated that patchy stomatal closure with a bimodal closure pattern may explain midday depression in top canopy leaves of a tropical rainforest (at Pasoh, Peninsular Malaysia). Moreover, application of numerical analyses to these canopy leaves shows that patchy bimodal stomatal closure is the main determinant of photosynthetic rate inhibition year round (Kosugi et al. 2009). Brodribb and Holbrook (2004) argued that hydraulic dysfunction at midday is linked to gas exchange depression, which may explain some of the spatial variation in stomatal aperture. However, it is still unclear whether patchy bimodal stomatal behavior actually occurs under field conditions (because the infiltration method is an all-or-nothing procedure that classifies groups of stomata as either open or closed depending on whether some or all of the stomata are above or below a particular threshold for liquid conductance), and whether the distribution of stomatal aperture on a single leaf (either uniform or non-uniform stomatal behavior) varies flexibly in response to stress factors within a single day.

To better understand the effects of stomatal dynamics on the midday depression of photosynthesis, we measured diurnal changes in leaf CO_2 exchange in 10 tree species; measurements were taken from middle to upper canopy layers in a tropical rainforest, and observations were made on individual stomatal apertures. We analyzed the relationships between diurnal changes in photosynthetic rate and patterns of stomatal aperture distribution on single leaves. We used a numerical procedure based on the Farquhar–von Caemmerer–Berry model (Farquhar et al. 1980) and Suzuki's Universal Micro-Printing (SUMP) method for stomatal observation.

Materials and methods

Site and materials

The study was conducted in a nursery at the Forest Research Institute Malaysia (FRIM), in Kepong, Peninsular Malaysia (3°23′N, 101°63′E). We measured in situ leaf gas exchange and observed the stomatal aperture distribution of leaves of potted trees (pot size: 18 cm diameter, 20 cm deep) of 10 species. The species selected were located at the middle and upper canopy levels (see Table 1). All species had heterobaric leaves. Data were collected from 29 January to 3 February 2010. Plants were watered several times during observations to prevent the soil from drying.

Measurement of leaf gas exchange

Diurnal changes in net assimilation rate (A, μmol m^{-2} s^{-1}) and stomatal conductance of water vapor (g_s, mol m^{-2} s^{-1}) of intact

Table 1. List of tree species of a Malaysian lowland tropical rainforest measured in this study. Height and ground diameter of each plant are shown.

Forest stratification	Family	Species	Height, cm	Ground diameter, cm	Measurement date
Upper layer	Dipterocarpaceae	Shorea leprosula	200	3.30	1 Feb. 2010
		S. macrantha	90	1.30	1 Feb. 2010
		S. hemsleyana	85	1.70	2 Feb. 2010
		S. sumatrana	180	2.90	1 Feb. 2010
		Dipterocarpus tempehes	175	2.00	2 Feb. 2010
		D. grandiflorous	200	2.70	2 Feb. 2010
Middle layer	Dipterocarpaceae	Hopea glaucescens	180	1.80	3 Feb. 2010
		H. subalata	185	1.30	3 Feb. 2010
		Vatica nitens	265	2.70	29 Jan. 2010
	Clusiaceae	Mesua ferrea	120	1.80	30 Jan. 2010

leaves were measured under natural photosynthetic photon flux density (PPFD, μmol m^{-2} s^{-1}), using an LI-6400 gas exchange measurement system (Li-Cor, Inc., Lincoln, NE, USA) with a 2 × 3 cm clear-top chamber. Gas exchange measurements were made on three leaves per plant. Photosynthetic photon flux density and leaf-to-air vapor pressure deficit (VPD, kPa) conditions on five measurement days are presented in Figure 1. Leaf temperature was 37.3 ± 4.1 °C (mean ± SD). The ambient CO$_2$ concentration was 397 ± 20 μmol mol^{-1} (mean ± SD).

Model description

We calculated normalized maximum rates of carboxylation at 25 °C (V_{cmax25}, μmol m^{-2} s^{-1}) to evaluate the effects of heterogeneous stomatal behavior on gas exchange, and to determine directly the apparent depression of photosynthetic capacity in the field (Takanashi et al. 2006). Maximum carboxylation rate (V_{cmax}) was calculated with a one-point method (Wilson et al. 2000, Kosugi et al. 2003, 2009, Grassi et al. 2005, Kosugi and Matsuo 2006, Takanashi et al. 2006), which is an inverse method based on the Farquhar–von Caemmerer–Berry model (Farquhar et al. 1980) that can be used to determine the actual responses of leaves in the field. In the one-point method, the apparent partial pressure of CO$_2$ within the intercellular space ($p(C_i)$*) is estimated from variables measured using a gas exchange methodology, and the 'apparent' normalized maximum carboxylation rate (V_{cmax25}*) is calculated from the values of A, $p(C_i)$* and leaf temperature (T_1). Here, we calculated V_{cmax25}*, assuming that the infinite internal conductance ($g_i = \infty$), and thus the CO$_2$ concentration in the chloroplast ($p(C_c)$) was the same as $p(C_i)$*. A possible influence of error in V_{cmax25} resulting from the estimation of g_i has been discussed by Takanashi et al. (2006) and Kosugi and Matsuo (2006). Although V_{cmax} is generally calculated assuming that $g_i = \infty$,

lower values of g_i (<0.1 CO$_2$ mol m^{-2} s^{-1}) affect the absolute value of V_{cmax25}. Thus, V_{cmax25}* was calculated as follows:

$$V_{cmax25}^* = (A + R_d) \frac{p(C_c) + K_c(1 + (p(O)/K_o))}{p(C_c) - (p(O)/2\tau)}$$
$$\times \frac{1 + \exp[(\Delta S(V_{cmax})(T_1 + 273) - \Delta H_d(V_{cmax}))/(R(T_1 + 273))]}{\exp[\Delta H_a(V_{cmax})(T_1 - 25)/(298R(T_1 + 273))]}$$

(1)

where R_d is the non-photorespiratory respiration rate (μmol m^{-2} s^{-1}), τ is the specificity factor of RuBisCo, $p(O)$ (21,000 Pa) is the partial pressure of O$_2$ at the sites of oxygenation, K_c and K_o are the Michaelis–Menten constants of RuBisCo for CO$_2$ and O$_2$, respectively, T_1 is leaf temperature (°C), R is the gas constant (8.31 J K^{-1} mol^{-1}), $\Delta H_a(V_{cmax})$ is the activation energy for V_{cmax}, $\Delta H_d(V_{cmax})$ is the deactivation energy for V_{cmax} and $\Delta S(V_{cmax})$ is an entropy term. The Arrhenius function was used to estimate the temperature dependence of parameters K_c, K_o, τ, V_{cmax} and R_d (detailed in Kosugi et al. 2003, Kosugi and Matsuo 2006, Takanashi et al. 2006).

To determine the effects of stomatal aperture distribution on gas exchange, we performed simulations to predict net assimilation rates at a given stomatal conductance for both uniform and patchy bimodal stomatal distributions (Takanashi et al. 2006). A patchy bimodal distribution indicates that whole-leaf conductance reflects either open or closed stomatal conductance. For patchy bimodal distribution, stomatal conductance for open and closed patches was g_{smax} and g_{smin}, respectively, and the open/closed patch ratio (r_{oc}) was determined from the observed g_s for a whole leaf. Net assimilation rate and intercellular CO$_2$ concentration for each patch were estimated with the Farquhar–von Caemmerer–Berry model used for determining patch stomatal conductance values. We assumed uniform photosynthetic parameters through the
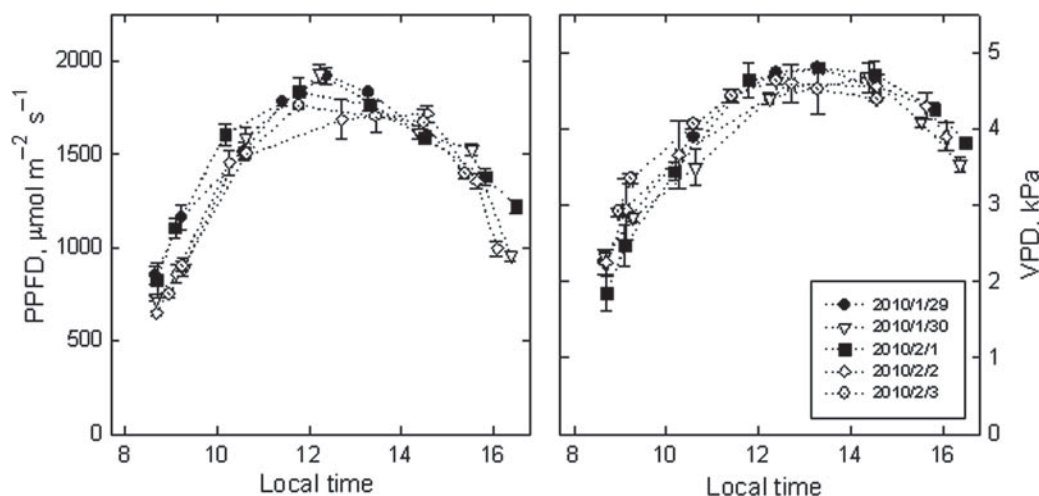

Figure 1. Averaged diurnal change in PPFD (μmol m^{-2} s^{-1}) and VPD (kPa) on each measurement day. Values are means ± SD.

whole leaf. The average net assimilation rate for the whole leaf was calculated by integrating net assimilation rates of individual patches.

In the model simulations, the net assimilation rate of each patch was calculated following Kosugi et al. (2003), Kosugi and Matsuo (2006) and Takanashi et al. (2006). Briefly, A of open (A_{op}) and closed (A_{cl}) patches were determined from the minimum value between the RuBP-saturated rate or carboxylation-limited net assimilation rate (A_c) and the electron transport- or RuBP regeneration-limited net assimilation rate (A_j), respectively, as follows:

$$A_c = V_{cmax25}$$
$$\times \frac{\exp[\Delta H_a(V_{cmax})(T_1 - 25) / (298R(T_1 + 273))]}{1 + \exp[(\Delta S(V_{cmax})(T_1 + 273) - \Delta H_d(V_{cmax})) / (R(T_1 + 273))]}$$
$$\times \frac{p(C_c) - p(O) / 2\tau}{p(C_c) + K_c(1 + p(O) / K_o)} - R_d. \tag{2}$$

$$A_j = \frac{J}{4} \times \frac{p(C_c) - (p(O) / 2\tau)}{p(C_c) + (p(O) / \tau)} - R_d. \tag{3}$$

Here V_{cmax25} is the intrinsic value that represents the carboxylation ability of leaves and is not always identical to the apparent value ($V_{cmax25}{}^*$), which exhibits significant diurnal fluctuations coupled with patchy stomatal closure (Takanashi et al. 2006). CO_2 concentration in the chloroplast of open patches ($p(C_c)_{op}$) was determined from A_{op} and g_{smax}, and that for closed patches ($p(C_c)_{cl}$) was determined from A_{cl} and g_{smin}. A J value was required to estimate the influence of depression in the electron transport rate on the net assimilation rate. To estimate J, we used optimal J_{max} values estimated from the relationship between V_{cmax} and J_{max} as follows:

$$J_{max} = k_j V_{cmax}. \tag{4}$$

The net assimilation rate (A_{leaf}) and CO_2 concentration in the chloroplasts ($p(C_c)_{leaf}$) of the whole leaf were calculated as follows:

$$A_{leaf} = r_{oc}A_{op} + (1 - r_{oc})A_{cl} \tag{5}$$

$$p(C_c)_{leaf} = r_{oc}p(C_c)_{op} + (1 - r_{oc})p(C_c)_{cl}. \tag{6}$$

The estimated maximum rate of carboxylation at 25 °C ($V_{cmax25}{}^*$) was determined by substituting values of A_{leaf} and $p(C_c)_{leaf}$ into equation (1).

For photosynthetic parameters, we used the observed optimal values of V_{cmax25} and g_{smax} of each species (listed in Table 2), and also used 0.0005 mol m^{-2} s^{-1} for g_{smin} and 2.2 for k_j. Based on the average line or the median value for evergreen

Table 2. List of parameters used in the biochemical photosynthetic model.

Species	V_{cmax25}, µmol m^{-2} s^{-1}	g_{smax}, mol m^{-2} s^{-1}
S. leprosula	48.9	0.28
S. macrantha	17.4	0.16
S. hemsleyana	26.7	0.08
S. sumatrana	16.1	0.09
D. tempehes	14.6	0.12
D. grandiflorous	20.3	0.10
H. glaucescens	17.3	0.05
H. subalata	21.0	0.06
V. nitens	10.9	0.09
M. ferrea	18.4	0.09

broadleaved trees listed by Kosugi and Matsuo (2006), we used the following parameterizations: 55,200 J mol^{-1} for ΔH_a (V_{cmax}), 220,000 J mol^{-1} for ΔH_d (V_{cmax}), 650 J mol^{-1} for ΔS (V_{cmax}) and 41,500 µmol m^{-2} s^{-1} for ΔH_a (R_d). Other parameters were as listed in Takanashi et al. (2006).

Observation of stomatal aperture distribution

To evaluate patchy stomatal behavior, we obtained the frequency distributions of stomatal aperture for single leaves of three top-canopy tree species (*Shorea leprosula* Miq., *Shorea macrantha* Brandis. and *Dipterocarpus tempehes* V.Sl.) from observations made using the SUMP method, which uses amyl acetate and thin celluloid plates (2 cm in diameter; SUMP Laboratory, Tokyo). Observations were made twice a day, once immediately following the onset of midday depression of A, and then halfway through the midday depression. Immediately after measuring leaf gas exchange, we made impressions of the surface of one of the three experimental leaves by fastening the celluloid plate dissolved by amyl acetate to the abaxial leaf surface (the site at which the gas exchange rate was measured). Then stomatal behavior was observed under a digital microscope (Model VH-Z450; Keyence, Osaka). A total of 35–40 microscopic fields per leaf (10–17 stomata in each field of vision) were observed. The widths of individual stomatal pores were determined from static images using NIH image software (National Institutes of Health, Bethesda, MD, USA). Because the leaves used for making impressions were destroyed, we used adjacent leaves with similar photosynthetic rates for leaf gas exchange measurements after treatment.

Results

Diurnal pattern of leaf CO_2 exchange

The averaged diurnal changes in PPFD (µmol m^{-2} s^{-1}) and VPD (kPa) on each measurement day are presented in Figure 1 ($n = 3$–9). The measurements were made on clear days.

Stomatal conductance (g_s) and A for all plant species varied within single days (Figure 2), and every leaf showed midday depressions of both parameters. Figure 3 compares the observed and simulated plots (simulated by numerical analysis based on the Farquhar–von Caemmerer–Berry model) of diurnal changes in A for three leaves of each plant species. The model assumes that the assimilation rate is affected by uniform or patchy bimodal distribution of stomatal aperture. In top-canopy species, the observed diurnal change in A was clearly depressed in late morning (Figure 3a). With the exception of S. leprosula and S. hemsleyana, observed A values recovered in the afternoons, but the rates were lower than morning rates. Midday depression of observed A also occurred in all sub-canopy species (Figure 3b). Observed A usually recovered in the afternoon, and for some species, increased to the morning level. Comparisons of observed and simulated A values clearly show that patchy bimodal distribution of stomatal aperture explains the severe depression of A, regardless of species, whereas uniform distribution of stomatal aperture explains optimum A in the early morning and recovery in the afternoon.

Distribution of stomatal aperture

Figures 4–6 present images (captured by the SUMP method) of individual stomatal apertures immediately following the onset of midday depression and during the midday depression; frequency distributions of stomatal aperture are also presented. In S. leprosula and S. macrantha, two peaks in the frequency distributions of stomatal aperture indicate that a patchy bimodal

distribution of stomatal aperture (observed stomata were either widely open or almost closed) occurred immediately following the onset of midday depression (Figures 4a and 5a). These bimodal distributions occurred within single isolated leaf patches, rather than between patches. In these two species, most stomata closed during midday depression (Figures 4b and 5b). A few stomata in each patch still remained open during the midday depression; however, between-patch differences were not apparent. In D. tempehes, the frequency distribution of stomatal aperture was bimodal during the midday depression (Figure 6b), but the frequency distribution immediately following the onset of midday depression was normal (Figure 6a). Between-patch differences were not observed.

Discussion

Midday depression in A clearly occurred in leaves of all observed plant species distributed in the upper and middle canopy layers. Numerical analysis showed that patchy bimodal stomatal closure largely explained this depression (Figure 3). Kosugi et al. (2009) also argued that patchy stomatal closure was the main factor limiting midday net assimilation in another top-canopy species (Dipterocarpus sublamellatus Foxw.) in a lowland dipterocarp forest in Peninsular Malaysia. The diurnal pattern in A differed slightly between plant species, but was similar among the three leaves of each species. Depressed values of A recovered to morning levels in sub-canopy tree species, but not in top-canopy tree species. In top-canopy
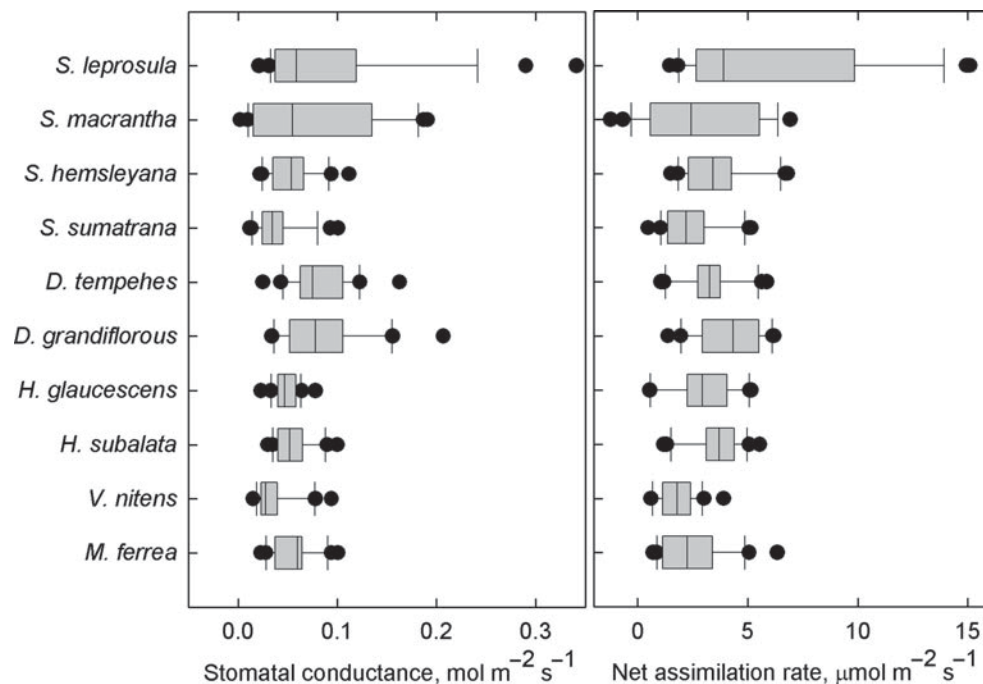
Figure 2. Variation in stomatal conductance (g_s) and net assimilation rate (A) for each plant species within single days (medians, 10th, 25th, 75th and 90th percentiles with error bars in box and whisker plots).
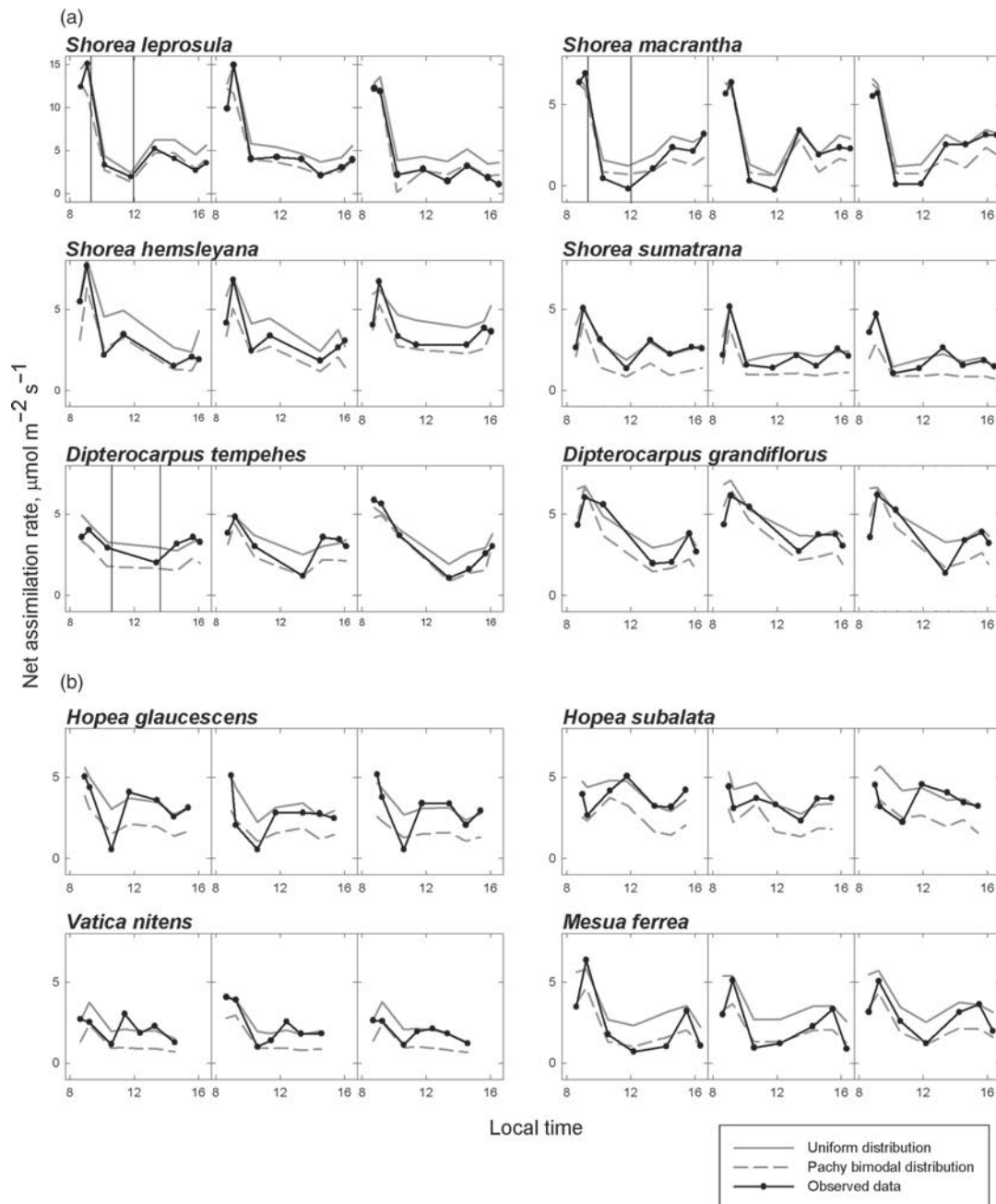
Figure 3. Diurnal changes in observed net assimilation rate (A), and simulation results assuming uniform stomatal closure and patchy bimodal stomatal closure [n = 3 leaves each of top-canopy species (a) and sub-canopy species (b)]. Solid vertical lines for *S. leprosula*, *S. macrantha* and *D. tempehes* indicate times when impressions were made of the abaxial leaf surfaces using the SUMP method.

conditions of tropical rainforests, tree leaves suffer severe environmental conditions through higher radiation, VPD and temperature than leaves in the lower forest layers (Chazdon et al. 1996, Kenzo et al. 2007). Thus, values of A in leaves of top-canopy tree species may be depressed more strongly than those of sub-canopy tree species.

Our numerical analysis demonstrated that patchy bimodal stomatal behavior occurred only during the midday depression, which strongly suggests that distribution patterns of stomatal aperture (uniform vs. non-uniform stomatal behavior) vary

flexibly within single days. Patchy stomatal closure might be induced by environmental factors (Kosugi et al. 2009) and/or circadian rhythms (Doughty et al. 2006). Kosugi et al. (2009) showed that severe midday depression of A and g_s on hot, sunny days is related to VPD, whereas moderate depression on cloudy and cool days is induced by circadian rhythms. In our study, all measurements were made on sunny, hot days. On those days, leaf temperature, VPD and thus transpiration rate increased quickly with radiation in the morning (Figure 1), followed by rapid and severe depression of A and g_s over the
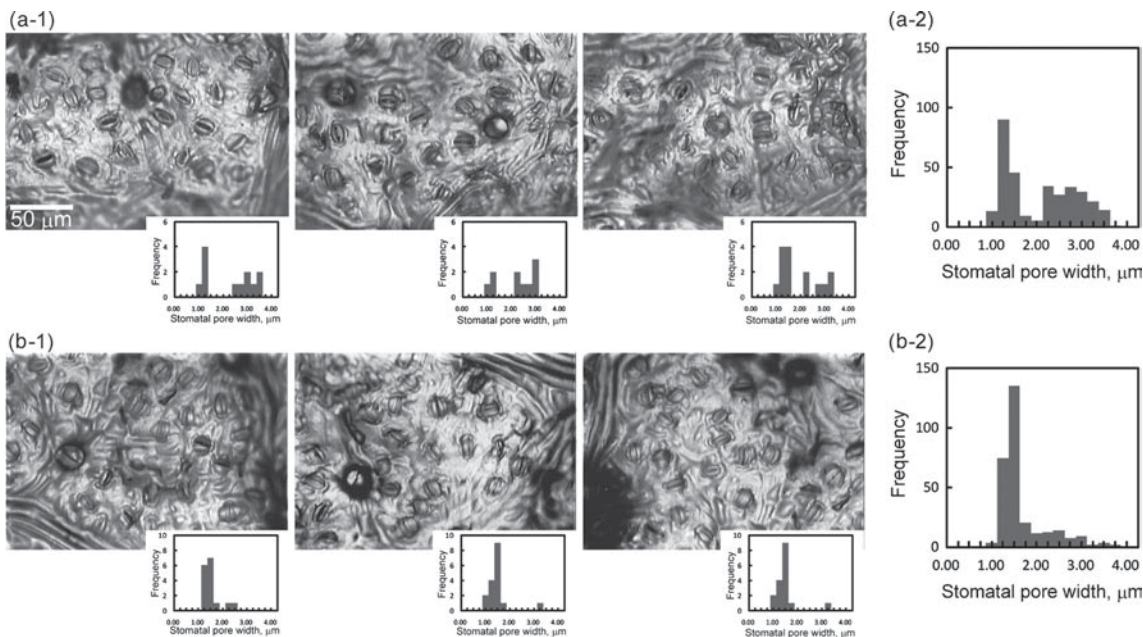
Figure 4. Images of individual stomatal behavior and frequency distribution of stomatal pore width (a-1) in leaves of *S. leprosula* immediately following the onset of midday depression (09:15) and (b-1) in the middle of the midday depression (12:00). Pooled frequency distribution data for stomatal pore width across times are shown to the right (a-2 and b-2).
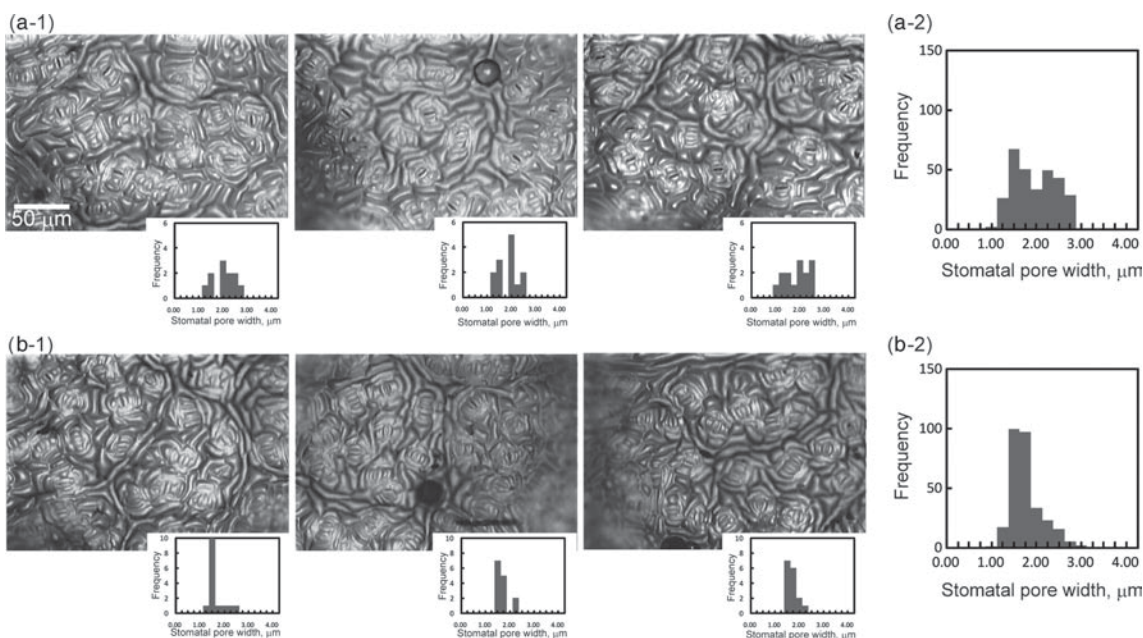

Figure 5. Images of individual stomatal behavior and frequency distribution of stomatal pore width (a-1) in leaves of *S. macrantha* immediately following the onset of midday depression (09:15) and (b-1) in the middle of the midday depression (12:00). Pooled frequency distribution data for stomatal pore width across times are shown to the right (a-2 and b-2).

midday period (Figure 3). Previous studies have reported strong correlations between midday depression of photosynthesis and VPD (e.g., Brodribb and Holbrook 2004, Tay et al. 2007). In most plant species observed, both A and g_s recovered in the afternoon (Figure 3), coupled with the depression of radiation and VPD (Figure 1). Our data suggest that

patchy bimodal stomatal closure occurs with increased VPD to prevent excess water loss from leaves through evapotranspiration, with concomitant severe depression of photosynthesis.

Patchy stomatal closure has been detected in some species with heterobaric leaves using the infiltration method (e.g., Beyschlang and Pfanz 1990, Küppers et al. 1999). However,
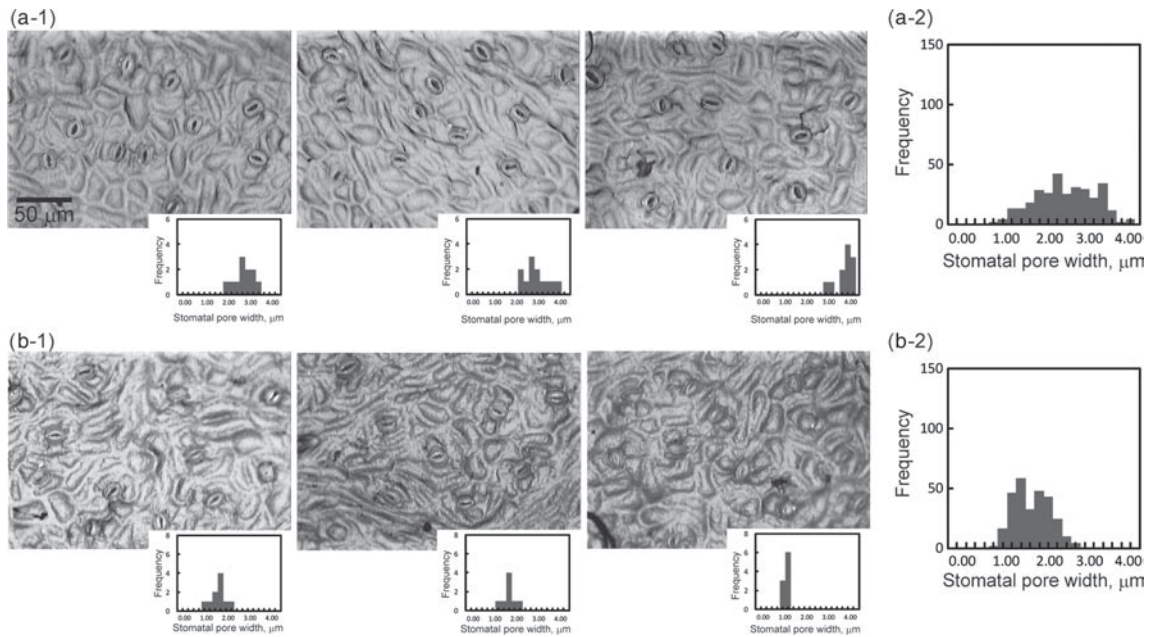
Figure 6. Images of individual stomatal behavior and frequency distribution of stomatal pore width (a-1) in leaves of *D. tempehes* immediately following the onset of midday depression (10:30) and (b-1) in the middle of midday depression (13:30). Pooled frequency distribution data for stomatal pore width across times are shown to the right (a-2 and b-2).

correlations between distribution of stomatal aperture and gas exchange characteristics remained unclear, because direct observation of individual stomatal movements under field conditions was technically difficult. Kaiser and Kappen (2000) observed in situ stomatal movements by video microscopy while simultaneously measuring leaf gas exchange in the field. They observed between 5 and 40 stomata in each experiment, but this methodology did not permit comparisons of stomatal aperture distribution on a single leaf. Our observation of patchy bimodal stomatal closure using the SUMP method allowed us to discern patterns of patchiness. We observed a bimodal stomatal closure pattern during midday depression of A in top-canopy leaves of the tropical rainforest species, *S. leprosula*, *S. macrantha* and *D. tempehes* (Figures 4–6). Although, bimodal distribution of stomatal aperture occurred within patches, rather than between patches. Within a bundle sheath extension (at least a small one having a few dozen stomata), lateral diffusion of CO_2 would be sufficiently rapid. Assuming uniform C_i within a patch, a bimodal distribution of stomatal aperture between patches is required to explain the midday depression of A induced by 'bimodal stomatal closure'. A bimodal frequency distribution of stomatal aperture in a small area within a patch would not cause serious overestimation of C_i (Cheeseman 1991). However, our data suggest that bimodal stomatal closure occurred among adjacent stomata within patches, rather than among patches within a single leaf, and still coupling with the depression of A induced by patchy stomatal closure that fits a bimodal frequency pattern. When the

pattern of stomatal closure is patchy bimodal, homogeneous behavior between adjacent stomata within a patch is predicted. A possible explanation for this is that the heterogeneity of C_i might occur within a patch, although the details remain unclear. Kamakura and Furukawa (2008) reported from direct observations that, in some cases, heterogeneous responses to CO_2 occur between adjacent stomata in leaves of homobaric species that lack bundle sheath extensions. Further analyses should take into account scale differences in the heterogeneity of stomatal behavior between and within patches and within single leaves.

We used saplings in this study, and it is possible that the behavior of these small trees differs from that of mature trees. Further studies of midday depression should be extended to mature trees.

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