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Hydraulically based stomatal oscillations and stomatal patchiness in *Gossypium hirsutum*

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Abstract. Slow stomatal oscillations (70–95 min), associated with feedback within the plant hydraulic systems, were studied in cotton (*Gossypium hirsutum* L.). Oscillations were only evident when the whole plant was exposed to light, and were not influenced by reductions in intercellular CO_2 concentrations (C_i) in intact, attached leaves. Oscillations were synchronised among different leaves of the same plant, even when the leaf-to-air vapour pressure difference (VPD) was reduced in a cuvette enclosing one of the leaves. In the trough phase of stomatal oscillations the apparent C_i was higher than expected from the combination of the observed assimilation rate and the $A(C_i)$ relationship measured in the absence of oscillations. Using chlorophyll fluorescence imaging we found evidence of stomatal heterogeneity in this phase. Finally, we found that stomatal oscillations appeared to be correlated with xylem embolism, with more vessels filled with gas at the peak than at the troughs of stomatal oscillations.

Keywords: chlorophyll fluorescence, photosynthesis, stomatal conductance, xylem embolism.

Introduction

Two major feedback loops, one involving the control of stomatal conductance (g_s) by intercellular CO_2 concentration (C_i) and the other involving the control of g_s by leaf water status, are thought to help stomata to adjust their conductance in accordance with the task of optimising and reconciling two opposing priorities, maximising CO_2 uptake for photosynthesis and minimising water loss via transpiration (Cowan and Farquhar 1977; Raschke 1979; Mott 1988).

Stomatal oscillations resulting from instabilities in the feedback control have been observed in several species (Barrs 1971; Farquhar and Cowan 1974). Oscillations with a period of the order of 20 min to an hour are thought to reflect instability of the feedback loop that controls stomatal aperture through the water content of the leaf (Cox 1968; Raschke and Kühl 1969). Most feedback components are designed to be negative, but Cox (1968) suggested that the 'hydropassive' movement of stomata by the epidermal pressure on the guard cells of stomata (Stålfelt 1929) acts as a positive feedback component of this loop, and plays a role in oscillations. It is also possible that delays in the signal transfer pathway can delay the negative feedback signal to the extent that the feedback becomes positive at the frequency of natural oscillations in stomatal aperture. Whatever the cause, it appears that at the frequency of natural oscillations, the increasing transpiration rate causes increasing stomatal conductance and faster increase in transpiration (and *vice versa*), instead of the stabilisation of transpiration (Farquhar 1973; Jarvis *et al*. 1999).

The term 'gain' is central in control theory, meaning the degree of change in the controllable parameter (e.g. stomatal aperture) in response to a unit change in the signalling parameter. The gain refers to the magnitude of a response,

Abbreviations used: A, net CO_2 assimilation rate; A_{calc}, A calculated from the $A(C_i)$ relationship; b_l, boundary-layer conductance to water vapour; C_i, intercellular CO_2 concentration; $C_{i(CAP)}$, capacity-weighted value or C_i adjusted from R and $C_{i(g)}$; $C_{i(g)}$, C_i calculated by the gas exchange system; E, transpiration rate; F_m and F_o, maximum and minimum fluorescence yields; F_m', chlorophyll fluorescence of a light adapted leaf; g_s, stomatal conductance to water vapour; IRGA, infrared gas analyser; q_N, non-photochemical quenching; R, fraction of leaf area inside the chamber where stomata remain open; VPD, leaf-to-air vapour pressure difference; Γ, CO_2 compensation point.

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and so describes fully a response with no delay (i.e. one that is very rapid). If the controllable parameter reacts slowly to the change in the signalling parameter, i.e. if the time dependence is significant, then we speak about a 'transfer function', a mathematical expression that involves the degree of change (gain), but also the time course of change of the controllable parameter. Control loops may arbitrarily be divided into several such transfer functions acting in series. The simplest model governing water-induced stomatal movements contains just two transfer functions: the physiological transfer function (H) indicates when and how much the stomatal conductance (g_s) changes in response to a unit change in the transpiration rate (E) and the 'environmental' transfer function or gain ($\partial E / \partial g_s$) indicates how much the transpiration rate (E) changes in response to a unit change in g_s (Farquhar and Cowan 1974). (One could think of H as describing signal transduction, and of $\partial E / \partial g_s$ as describing the sensitivity of the controller.) A large total loop gain — the product of the component gains — is the main factor inducing stomatal oscillations. Consequently, any perturbation of the loop involving the hydraulics and stomata can initiate oscillations if the overall gain of the feedback loop is high enough. In other words, if the loop gain is sufficiently large, then the oscillations will grow until they become limited by a non-linearity (ceiling) in a transfer function. Cowan (1972) gave as examples the maximum stomatal aperture limited by stomatal anatomy, or the lower limit in transpiration imposed by closed stomata. If the loop gain is not sufficiently large, the response to a perturbation is damped oscillations. The actual period of oscillations is determined in part by the interplay of delays in the system (Farquhar 1973; Farquhar and Cowan 1974).

Leaf-to-air vapour pressure difference (VPD) is the major contributor to the environmental gain ($\partial E / \partial g_s$). A given change in g_s causes a bigger change in E when VPD is large. Root hydraulic resistance, however, is an important factor contributing to the gain of the physiological (hydraulic) transfer function (H). Lowering root temperature, for example, increases root resistance, which causes a drop in both water potential and stomatal aperture after a positive perturbation in E.

In addition to root resistance, cavitation of xylem vessels at any point along the water pathway, from root to leaf, also increases the gain of the physiological transfer function (H). Therefore, embolism is a potentially important process influencing plant hydraulic conductance, loop gain, and the onset of oscillations. Although refilling of embolised xylem vessels may occur over short time scales (Canny 1998; Holbrook and Zwieniecki 1999), the interaction between stomatal oscillations and xylem embolism has not yet been examined. The potential relevance of embolisms is exemplified by the observations in stems of *Laurus nobilis,* where refilling of embolised vessels can occur in 20 min (Salleo *et al*. 2004), a time scale consistent with stomatal oscillations.

Thus, the main objectives of our study were to (i) assess effects of plant age, light, C_i and VPD on stomatal oscillations, including their loop gain (damping) and frequency, (ii) relate stomatal oscillations to stomatal patchiness (heterogeneity in stomatal aperture) by using chlorophyll fluorescence imaging, and (iii) assess the role of embolisms in the hydraulic transfer function by measuring whether the xylem vessels in petioles embolise during oscillations.

Materials and methods

Plant material

Cotton plants (*Gossypium hirsutum* L. cv. 'Deltapine 16') at three different stages of development (pre-flowering, 45 d; flowering, 60 d; and boll-growing plants, 80 d) were used in this study. For each stage of development three plants were used. The plants were grown in 10-dm^3 pots in a sunlit glasshouse (mean maximum light intensity of 700 μmol m^{-2} s^{-1}) at Canberra, Australia. The pots were filled with a sterilised mixture of mushroom compost, sand, peat and perlite (2 : 2 : 1 : 1.5), amended with 20 g per pot of a slow-release fertiliser (Osmocote Plus, Heerlen, Holland). The plants were watered twice a day to keep the soil near field capacity. Night / day air temperature was 20 / 25°C with a daytime relative humidity of ~60%.

Leaf gas-exchange parameters

Net CO_2 assimilation rate (A), stomatal conductance to water vapour (g_s) and transpiration rate (E) were measured with portable open gas-exchange systems fitted with infrared gas analysers (IRGA, LI-6400, Li-Cor, Lincoln, NE). Data were collected from May to July 2004 on fully expanded leaves of similar age and appearance. Gas exchange data were collected every 120 or 180 s by the LI-6400 on autolog mode. In the chamber, the leaf was illuminated with a red–blue light source (Li-6400-02B). The airflow rate was kept constant during the experiment, and humidity within the chamber (and hence the leaf-to-air VPD) was determined by controlling the proportions of moist and dry air before the inlet to the chamber (i.e. the system was not set to control the humidity within the chamber, and humidity varied as a function of the transpiration rate). Within the chamber (6 cm^2) the airflow rate was set constant at 400 μmol s^{-1} and the resulting boundary-layer conductance (b_l) to the diffusion of water vapour was 2.84 mol m^{-2} s^{-1}. This conductance was much greater than that occurring when the leaves were outside the chamber, 0.33–0.4 mol m^{-2} s^{-1}, determined by measuring water loss from a wet blotting paper of the same size and shape as a cotton leaf, together with the temperature of the blotting paper and the vapour pressure in the air.

Dependence of oscillations on plant ontogeny and the proportion of open stomata in patches

To assess effects of plant ontogeny, data were collected from well-watered plants at pre-flowering (45 d), flowering (60 d) and boll-growth stages (80 d). In the troughs of oscillations stomatal conductance became patchy and the C_i calculated from gas-exchange chamber averages of A and E (the g_s-weighted value or $C_{i(g)}$, Farquhar 1989) no longer followed the steady-state $A(C_i)$ curve. We assume that in the mesophyll under the open stomata the $A(C_i)$ relationship was still the same as in the steady state with all stomata open. Therefore, we calculated the proportion of open stomata (R) from the following relationship (Downton *et al*. 1988):

$$C_{i(CAP)} = [(1 - R)\Gamma + C_{i(g)}R],$$

where $C_{i(CAP)}$ is the capacity-weighted C_i (Farquhar 1989), Γ is the CO_2 compensation point, R, the fraction of leaf area within the chamber where stomata remain mostly open ($R = A / A_{calc}$). A_{calc} was estimated

using data from an $A(C_i)$ curve, previously generated in the same leaf at steady-state conditions (g_s, 0.16–0.27 mol m^{-2} s^{-1}), as: $A_{calc} = \alpha(C_i - \Gamma)$, where α is the slope of the steady-state $A(C_i)$ curve.

Diurnal changes and the effect of plant illumination on oscillations

To assess circadian effects on stomatal oscillations, data were collected during day (0900–1800 hours) and night (1900–0400 hours) while the leaf in the Li-Cor chamber was illuminated constantly with a PPFD of 1000 µmol m^{-2} s^{-1} and the rest of the plant shoot kept under a moderate light intensity of 200 µmol m^{-2} s^{-1} (or in the dark in some experiments). In the chamber, leaf temperature was 22–24°C. Incoming $[CO_2]$ into the leaf cuvette was 380 µmol mol^{-1}. Relative humidity was 30–35% (for high leaf-to-air VPD) and 70–75% for low VPD. Oscillations in stomatal aperture were triggered by turning the light on.

Effect of $[CO_2]$ on oscillations

Gas-exchange characteristics were recorded during oscillations at a mean leaf temperature of 23°C and constant irradiance while incoming $[CO_2]$ into the leaf chamber was reduced gradually from 380 to 40 µmol mol^{-1}. This was done to reduce variations in C_i and hence break any feedback loop involving C_i.

Synchronous oscillations

To test whether stomatal oscillations occur synchronously in different leaves of the same plant, two gas-exchange systems were attached to different leaves of similar age and appearance on the same plant. PPFD and leaf temperature were 473 µmol m^{-2} $s^{-1}$and 23°C. The CO_2 concentration of air entering the leaf chambers was kept constant at 380 µmol mol^{-1}. A third leaf, subject to the same conditions, was attached to an imaging-PAM chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) to record fluorescence data.

In addition, to assess the effect of vapour pressure (VP) on stomatal oscillations, in one of the leaves attached to an IRGA, air VP was gradually decreased from 2.4 to 1.2 kPa until midday and then increased again in the afternoon.

Chlorophyll fluorescence

Images of chlorophyll fluorescence were recorded with an imaging-PAM chlorophyll fluorometer (Heinz Walz GmbH) on a leaf area of 26×34 mm^2. Chlorophyll fluorescence parameters (maximum fluorescence, F_m; minimum fluorescence, F_o) were recorded on leaves that had been dark adapted for 20 min, whereas F_m' (the F_m value of light-adapted leaves) was measured subsequently under actinic light. Incident actinic light was 473 µmol m^{-2} s^{-1} and the saturation pulse was 2400 µmol m^{-2} s^{-1} (470 nm). Actinic light and the saturation pulse in the PAM-system were generated by a ring of 96 LEDs. Gas-exchange and fluorescence data were collected simultaneously to assess effects of stomatal oscillations on maximum chlorophyll fluorescence in a light-adapted leaf (F_m'). Images of chlorophyll fluorescence were also recorded to observe effects of stomatal oscillations on stomatal patchiness.

Xylem embolism

Petiole samples were collected from the same plant (but from different leaves) being monitored by gas exchange. Petiole samples were collected at four stages during an oscillation period: peak, trough, and intermediate points between peak and trough phases. Removal of some leaves did not stop oscillations because these leaves represented a small fraction of plant foliage. Each sample was frozen on the plant by clamping a segment of the petiole between the jaws of cryo-pliers that had been previously cooled in liquid nitrogen. The frozen samples were cryo-stored in liquid nitrogen until they could be prepared for examination by cryo-scanning electron microscopy (cryo-SEM). A small segment of the sample was mounted in a slot in an aluminium stub with a mixture (1 : 1) of colloidal graphite and tissue-freezing medium (ProSciTech, Thuringowa, Queensland, Australia). A transverse section of the petiole segment was planed with a glass knife in a cryo-microtome at −90°C. The sample was etched in the stage of a cryo-scanning electron microscope (Cambridge S-360, Cambridge Instruments, Cambridge, UK) for a few minutes starting at −150°C and ending at −90°C to reveal cell outlines. It was coated with 10 nm of gold and observed at 15 kV. Micrographs were taken with a SEM digital imaging system. On nine petioles (from the same plant), xylem tissue was assessed visually to determine relative fractions of vessels that were filled with either water or gas. On average 142 vessels (above 25 µm in diameter) per petiole were examined to determine the proportion of gas-filled vessels. The experiment was conducted twice with similar results.

Results

Dependence of oscillations on plant ontogeny and diurnal variation in stomatal oscillation

Stomatal oscillations were observed in plants at the flowering stage and at the beginning of the boll-growing period, but not in those examined at the pre-flowering stage (Fig. 1). When lights were turned on in the morning, steady oscillations of assimilation rate and stomatal conductance were observed in the morning (Fig. 2). The oscillations continued all day, but their amplitude tended to decrease at the end of the afternoon (Fig. 2). The same trends were observed in C_i (Fig. 2*A*). VPD consistently increased as stomatal humidification of the chamber declined (Fig. 2*B*).

During the day, carbon assimilation (A) oscillated between 11 and 15 µmol m^{-2} s^{-1} in the morning, with

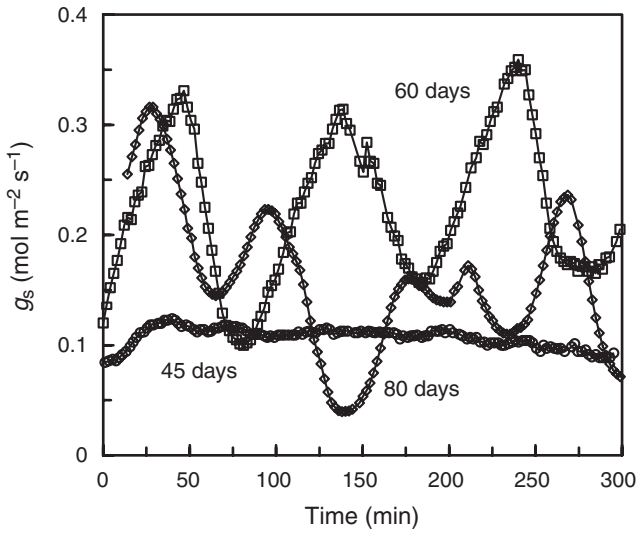

Fig. 1. Time course of stomatal conductance in leaves of cotton plants at the vegetative (45 d, ○), flowering (60 d, □), and post-flowering period (80 d, ◇). In the leaf cuvette irradiance was 1000 µmol m^{-2} s^{-1}. Incoming $[CO_2]$ into the leaf chamber was kept at 380 µmol mol^{-1}. Whole plant under moderate light intensity (200 µmol m^{-2} s^{-1}) and average leaf temperature of 23°C. The experiment was conducted twice with similar results.

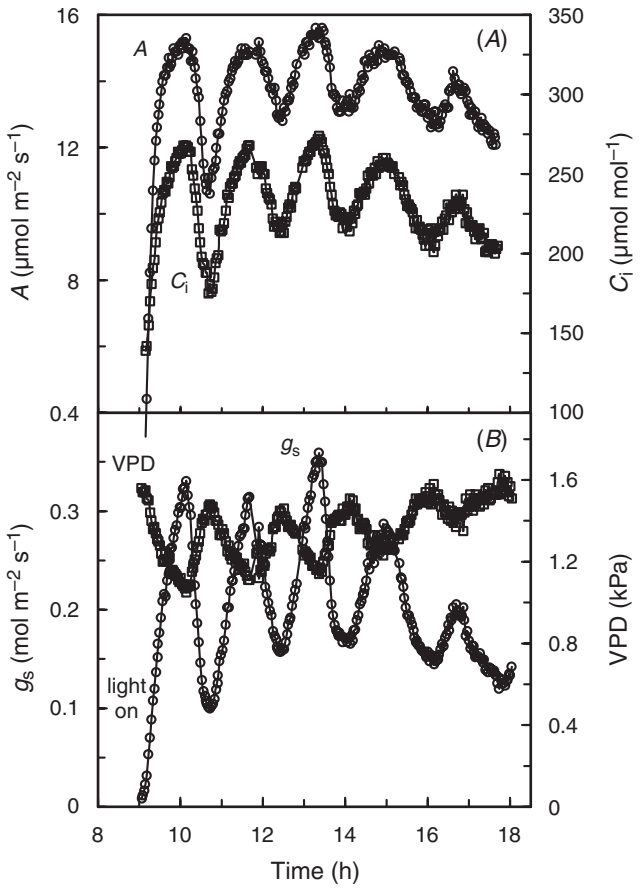

Fig. 2. Oscillations in carbon assimilation (A, ○) and intercellular CO_2 concentration (C_i, □) (A), and stomatal conductance (g_s, ○) and leaf-to-air vapour pressure difference (VPD, □) (B) on a cotton leaf. In the leaf cuvette, boundary layer conductance was 2.8 mol m^{-2} s^{-1} and irradiance 1000 μmol m^{-2} s^{-1}. Whole plant was kept under moderate light intensity (200 μmol m^{-2} s^{-1}). [CO_2] and leaf temperature as described in Fig. 1. The experiment was conducted twice with similar results.

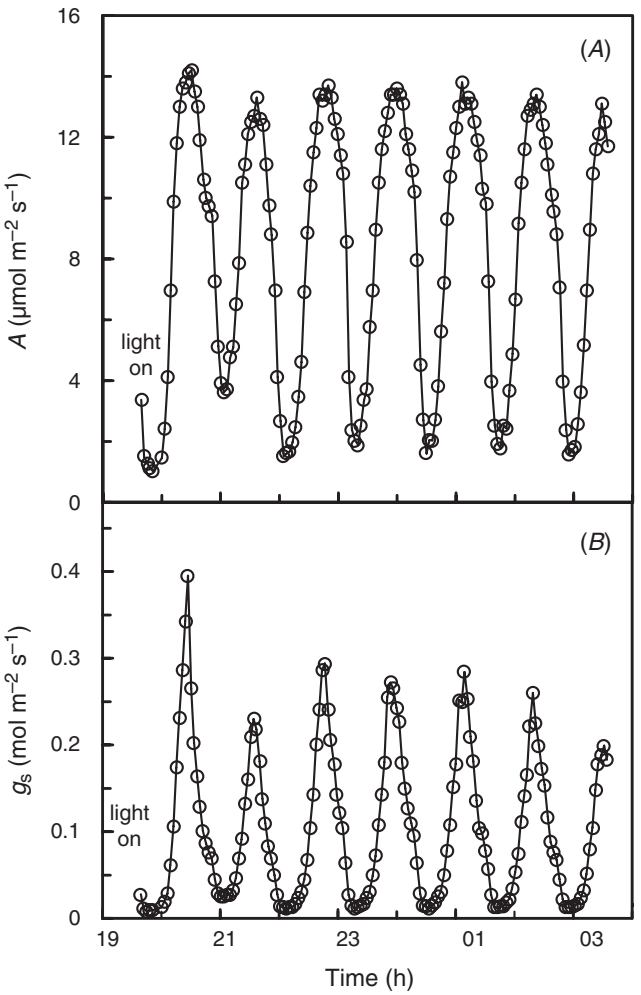

Fig. 3. Sustained oscillations in (A) carbon assimilation (A) and (B) stomatal conductance (g_s) in a cotton leaf. The experiment was conducted at night, twice, with similar results. Plant environment as described in Fig. 2.

a lower amplitude in the afternoon (Fig. 2*A*). Stomatal conductance (g_s) oscillated from 0.1 to 0.36 mol m^{-2} s^{-1}, with a mean value of around 0.23 mol m^{-2} s^{-1}, and an oscillation period of 96 min (Fig. 2*B*). Changes in g_s were in phase with those in A. During oscillations, increase in g_s led to a decrease in leaf-to-air VPD for two reasons. First, leaf temperature decreased from ~23.5 to 22.5°C as transpiration rate increased with the opening of stomata, and, second, increased E caused absolute humidity to increase by ~2 mmol mol^{-1}.

Influence of plant illumination on stomatal oscillations at night

At night, with the whole plant exposed to light, A and g_s oscillated consistently over well-defined periods of 71 min. In this experiment, A oscillated from 1.5 to 13.5 μmol m^{-2} s^{-1} (Fig. 3*A*) and g_s oscillated from 0.015 to 0.3 mol m^{-2} s^{-1} (Fig. 3*B*). As observed during the day, C_i also oscillated in concert with g_s, from 50 to 275 μmol mol^{-1} at the highest g_s value (data not shown). At the trough phases of oscillation periods, A was lower than predicted from the steady-state $A(C_i)$ curve (Fig. 4). Based on this discrepancy and using the $A(C_i)$ relationship from Fig. 4, we estimate that stomata remained open during the trough phase in 13 to 55% of the leaf area (Table 1). Also, it is worth noting that at the troughs, $C_{i(CAP)}$ was only 56% of $C_{i(g)}$ values calculated assuming the stomata were equally open across the leaf (Table 1; Fig. 5).

When the whole plant (except for the illuminated leaf in the cuvette) was kept in the dark for the whole night, the oscillations were damped (data not shown). A and g_s peaked following exposure of the leaf to light, but after a short period, this initial oscillation rapidly diminished and A and g_s remained constant at ~8 μmol m^{-2} s^{-1} and 0.065 mol m^{-2} s^{-1}, respectively, until midnight. However,

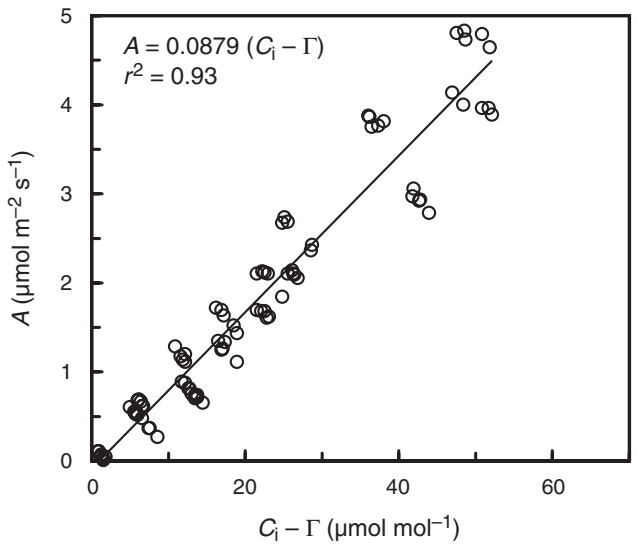

Fig. 4. $A(C_i)$ response curve in a cotton leaf at an irradiance of 1000 μmol m^{-2} s^{-1}, leaf temperature of 23°C, and boundary layer conductance of 2.84 mol m^{-2} s^{-1} inside the leaf chamber. Data were collected at daytime (1200–1600 h) in two fully expanded leaves of similar age and appearance and after observing for 30 min that stomatal conductance was at steady state at ambient [CO_2].

after that, g_s tended to increase to 0.08 mol m^{-2} s^{-1} as dawn approached.

Effect of C_i on oscillations

Decreasing C_i from 250 to 40 μmol mol^{-1} had no measurable effect on either stomatal oscillations or the oscillation period, the latter being ~70 min in duration (Fig. 5). However, when C_i was depleted, the opening phase lasted only 22 min instead of 30–33 min as observed under ambient [CO_2]. The assimilation rate tended to oscillate, but it damped over time as C_i was reduced within the leaf. It is also worth noting that at the trough phase the percentage of area where stomata remained open was 22% (trough 8, Table 1), and, as noted above, C_i values estimated from IRGA measurements ($C_{i(g)}$) were almost double the adjusted values, ($C_{i(CAP)}$ line in Fig. 5).

Synchronous oscillations

Stomatal conductance and assimilation rates oscillated in concert in different leaves from the same plant (Fig. 6). Reducing the VPD in the cuvette of leaf 1 up to midday (Fig. 6*B*) did not stop oscillations. In addition, g_s reached higher values (up to 1 mol m^{-2} s^{-1}) at lower VPD (around midday, Fig. 6*B*) than at higher VPD (after 1400 in leaf 1 Fig. 6*B*). Oscillations were nearly sinusoidal during the morning (leaf 2, Fig. 6*C*) and at night (Fig. 3), but they damped and died away in the afternoon (Fig. 6*C*).

Stomatal patchiness, reflected by heterogeneities in maximum fluorescence in light-adapted leaved (F_m'), was related to stomatal oscillations. More patchiness was observed at the trough phase of oscillations when g_s values were lower (Fig. 7).

Xylem embolism

There was considerable temporal variation in the proportion of vessels embolised, with the greatest proportion being at times of peak transpiration rate, and the least during the troughs (Figs 8, 9).

Discussion

Dependence of oscillatory occurrence on plant ontogeny

The 45-d-old plants were ~28 cm tall and had nine fully developed leaves, whereas the 60-d-old plants were ~65 cm tall and had ~40 leaves. We suggest that the absence of stomatal oscillations in leaves of young plants (45 d) is probably associated with the relatively small leaf area in relation to the water uptake capacity of the root system. That is, there was little gain involving the hydraulic system (low *H*). In this case, it should be expected that even a large environmental gain (e.g. a rather high VPD) would be insufficient to cause a large overall loop gain, and this would make the plant less prone to exhibiting oscillatory behaviour (Farquhar *et al*. 1978). Barrs and Klepper (1968) reported that

Table 1. Assimilation rate measured (A_{meas}) and calculated (A_{calc}), capacity-weighted C_i or $C_{i(CAP)}$ calculated from gas-exchange data ($C_{i(g)}$) and the fraction of leaf area where the stomata remain open, *R*

A_{calc} was obtained from Fig. 4 [$A = 0.0879\ (C_i - 48)$]; $C_{i(CAP)} = (1 - R)\Gamma + C_{i(g)}\ R$. Values are mean ± s.e., $n = 4$–6. The time and trough phases correspond to Fig. 3, except trough 8, which corresponds to Fig. 5

Time	Trough phase	A_{meas} (μmol m^{-2} s^{-1})	A_{calc} (μmol m^{-2} s^{-1})	$C_{i(g)}$ (μmol mol^{-1})	$C_{i(CAP)}$ (μmol mol^{-1})	R (A_{meas} / A_{calc})
1936–1951 hours	1	1.27 ± 0.09	10.2 ± 1.11	164 ± 13	62 ± 1	0.13 ± 0.02
2101–2107 hours	2	3.76 ± 0.06	7.32 ± 0.67	131 ± 8	96 ± 1	0.53 ± 0.06
2204–2219 hours	3	2.12 ± 0.33	8.33 ± 0.92	142 ± 11	69 ± 4	0.24 ± 0.06
2311–2323 hours	4	2.58 ± 0.40	5.45 ± 1.07	110 ± 12	77 ± 4	0.55 ± 0.11
0030–0042 hours	5	2.48 ± 0.38	7.53 ± 1.33	133 ± 15	75 ± 4	0.38 ± 0.09
0143–0200 hours	6	3.41 ± 0.81	7.07 ± 1.23	128 ± 14	76 ± 9	0.55 ± 0.13
0255–0313 hours	7	3.35 ± 0.92	7.75 ± 0.95	136 ± 11	73 ± 10	0.50 ± 0.16
0405–0420 hours	8	1.16 ± 0.23	5.65 ± 0.63	112 ± 7	61 ± 3	0.22 ± 0.04

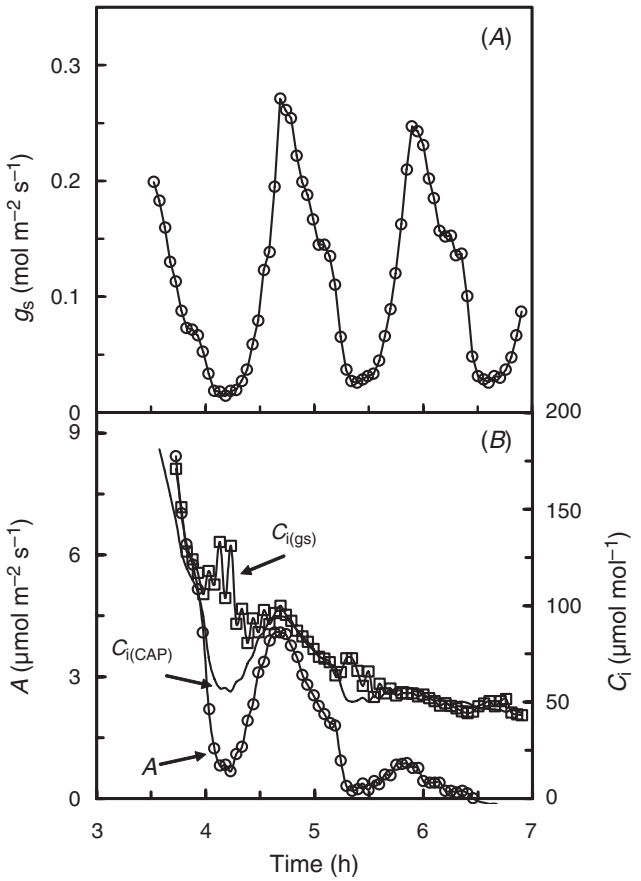

Fig. 5. Oscillations in stomatal conductance (g_s, ○) (A), and carbon dioxide assimilation (A, ○) and C_i calculated by gas exchange ($C_{i(g)}$, □) in a cotton leaf as a function of time (B) at gradually decreased ambient concentrations of CO_2. C_i adjusted values ($C_{i(CAP)} = [(1 - R)\Gamma + C_{i(g)} R]$ are also shown (solid line).The experiment was conducted with similar results. Leaf irradiance inside the chamber was kept constant at 1000 μmol m^{-2} s^{-1}.

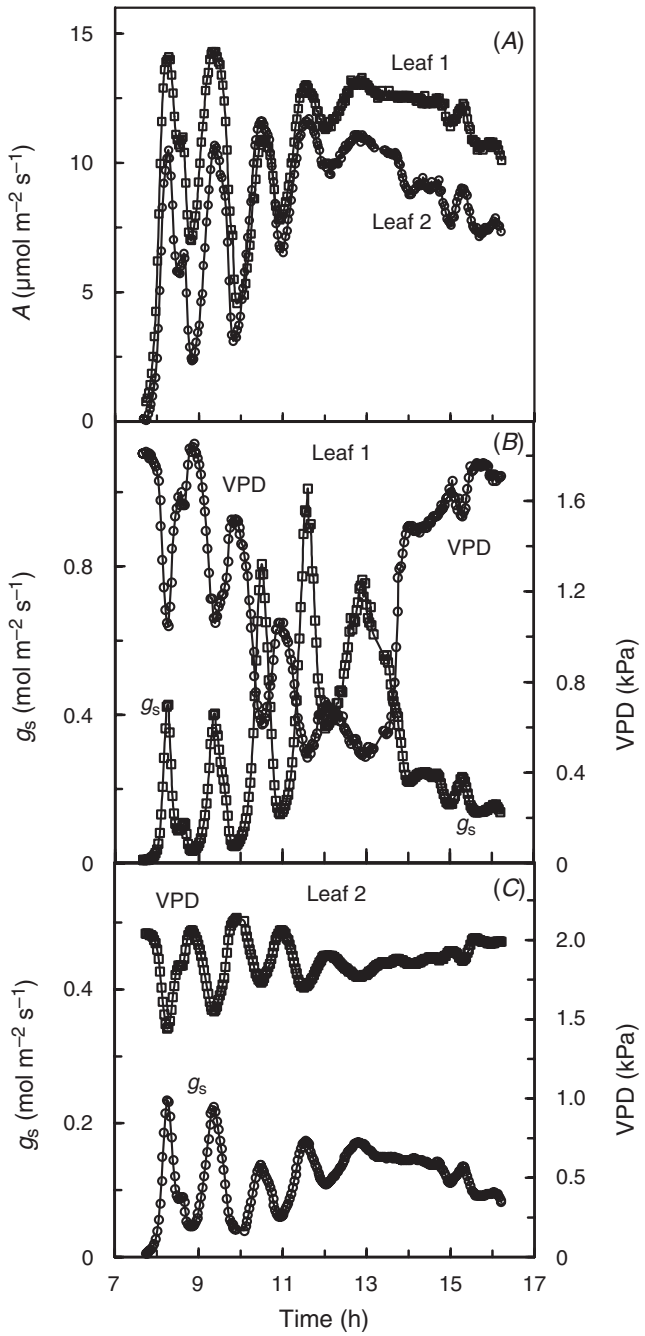

Fig. 6. Unsustained oscillation in carbon assimilation (A), stomatal conductance (g_s), and leaf-to-air VPD in two cotton leaves (each of them monitored with a different IRGA system) measured simultaneously. Irradiance within the leaf chamber was 473 μmol m^{-2} s^{-1} while the whole shoot received an irradiance of 200 μmol m^{-2} s^{-1}. The experiment was conducted twice with similar results. Carbon assimilation in both leaves (A) and g_s in leaf 1 (B) and leaf 2 (C). [CO_2] and leaf temperature as described in Fig. 1.

reducing plant foliage by defoliation reduced the occurrence of oscillations.

Diurnal variation in stomatal oscillations

Stomatal conductance and rates of photosynthesis oscillated in cotton over periods ranging from 70 to almost 100 min, which is slower than cyclic oscillations in stomatal conductance previously reported in cotton (Farquhar and Cowan 1974) or in other species (Barrs 1971). There was a tendency (Fig. 2) for the period to increase with time in the photoperiod. Farquhar (1973) reported that the periodicity in cotton increased from 43 min for the first oscillation to 63 min for the last of the photoperiod. The presence of steady oscillations in the morning and damped oscillations in the afternoon (Fig. 2*B*, but particularly Fig. 6) presumably reflected reduction in the overall loop gain. Farquhar and Cowan (1974) observed that oscillations occurred when the environmental gain was greater than 20.2 mmol mol^{-1}. However, in the present experiment humidity varied only with transpiration rate, and consequently the environmental gain ($\partial E / \partial g_s$) showed little trend to decrease during the day, remaining at ~1.6 kPa [i.e. $\partial E / \partial g_s$ of 17.6 mmol (H_2O) mol^{-1}(air), in terms of humidity difference, Fig. 2*B*].

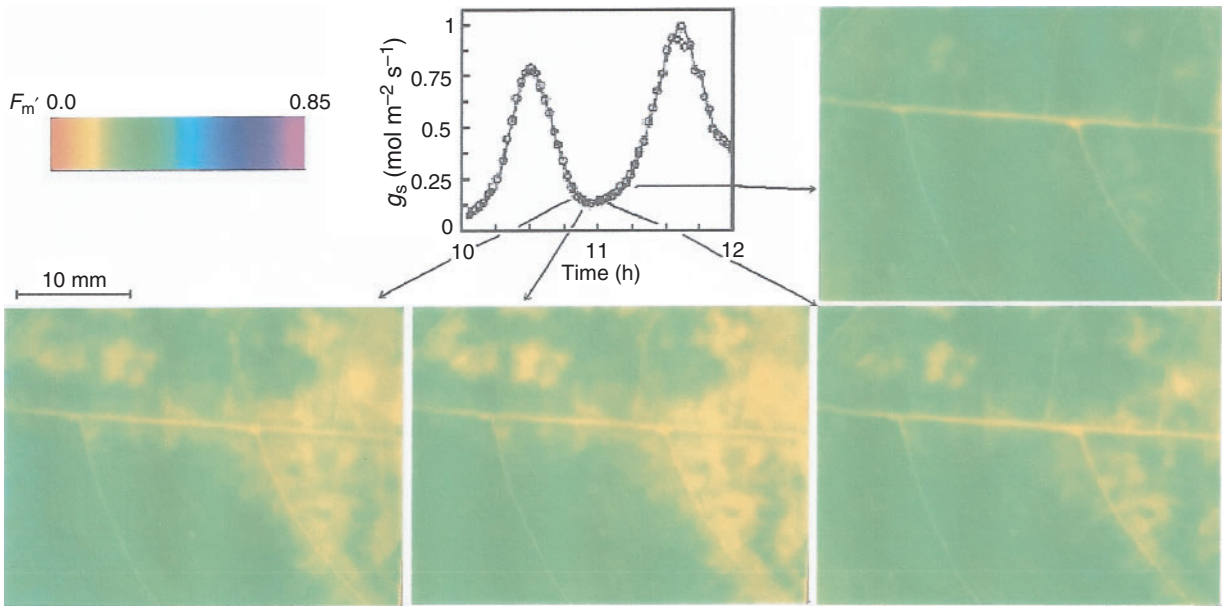

Fig. 7. Imaging of chlorophyll fluorescence recorded in a cotton leaf exhibiting oscillations in stomatal conductance (g_s). Maximum fluorescence yield in a light adapted leaf (F_m') was heterogenous at the trough phase of stomatal oscillations. The experiment was conducted twice with similar results.

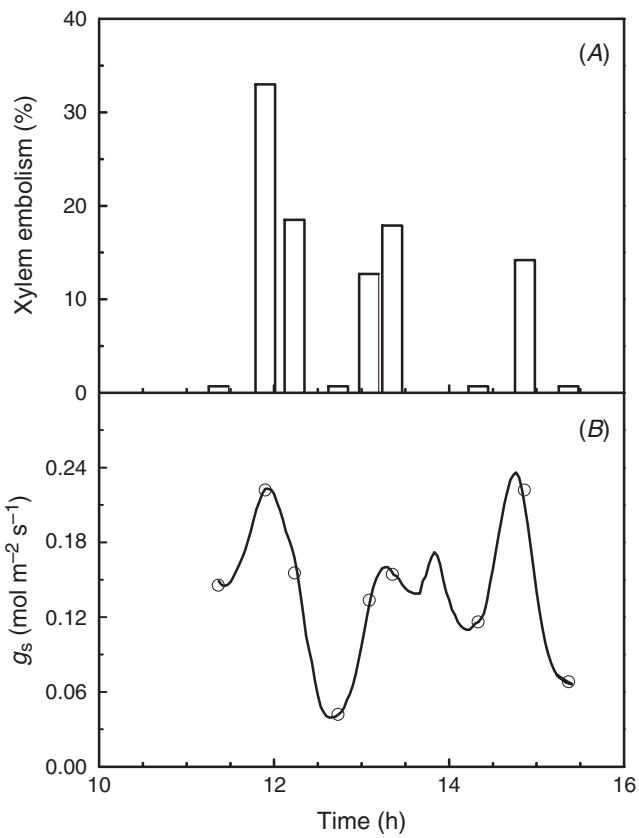

Fig. 8. Occurrence of gas-filled xylem vessels in petioles of cotton leaves (*A*) from the same plant in relation to oscillations in stomatal conductance (g_s) (*B*). Symbols in (*B*) show the times when petioles were collected to be examined for xylem embolism. The experiment was conducted twice with similar results.

Thus, it is likely that it is the gain of the hydraulic transfer function that is decreasing during the day. A diurnal rhythm in root resistance of cotton has been documented (Barrs and Klepper 1968; Henzler *et al*. 1999; Clarkson *et al*. 2000), with root resistance being high at night. It may explain our observation that stomatal conductance and *A* were lower in experiments carried out at night than during the day (Figs 2, 3). The observation that leaf gas exchange is influenced by circadian rhythms has been made in several other species, such as *Tradescantia virginiana* (Martin and Meidner 1971), *Vicia faba* and *Commelina communis* (Meidner and Willmer 1993), and *Saururus cernuus* (Williams and Gorton 1998).

Influence of plant illumination on oscillations

Sustained oscillations observed in illuminated plants at night (Fig. 3*B*) indicate a greater instability in the closed-loop system at night. We can infer that root resistance, an important component of *H*, remained high during the whole night. Root resistance is an important contributor to *H* because a greater resistance means that a positive perturbation of *E* causes a greater drop in water potential across the root and then a greater drop in g_s. This result is consistent with the findings of Kanemasu and Tanner (1969), who observed that stomatal oscillations occurred only at night in illuminated leaves.

Longer periods of stomatal oscillation (exceeding 30 min), as observed in our experiments, have been associated with perturbations of the plant hydraulic system (Raschke and Kühl 1969; Barrs 1971). The fact that resistance to water flux also occurs in plants growing in nutrient solutions (Barrs and Klepper 1968; Farquhar 1973) is not surprising as it is thought

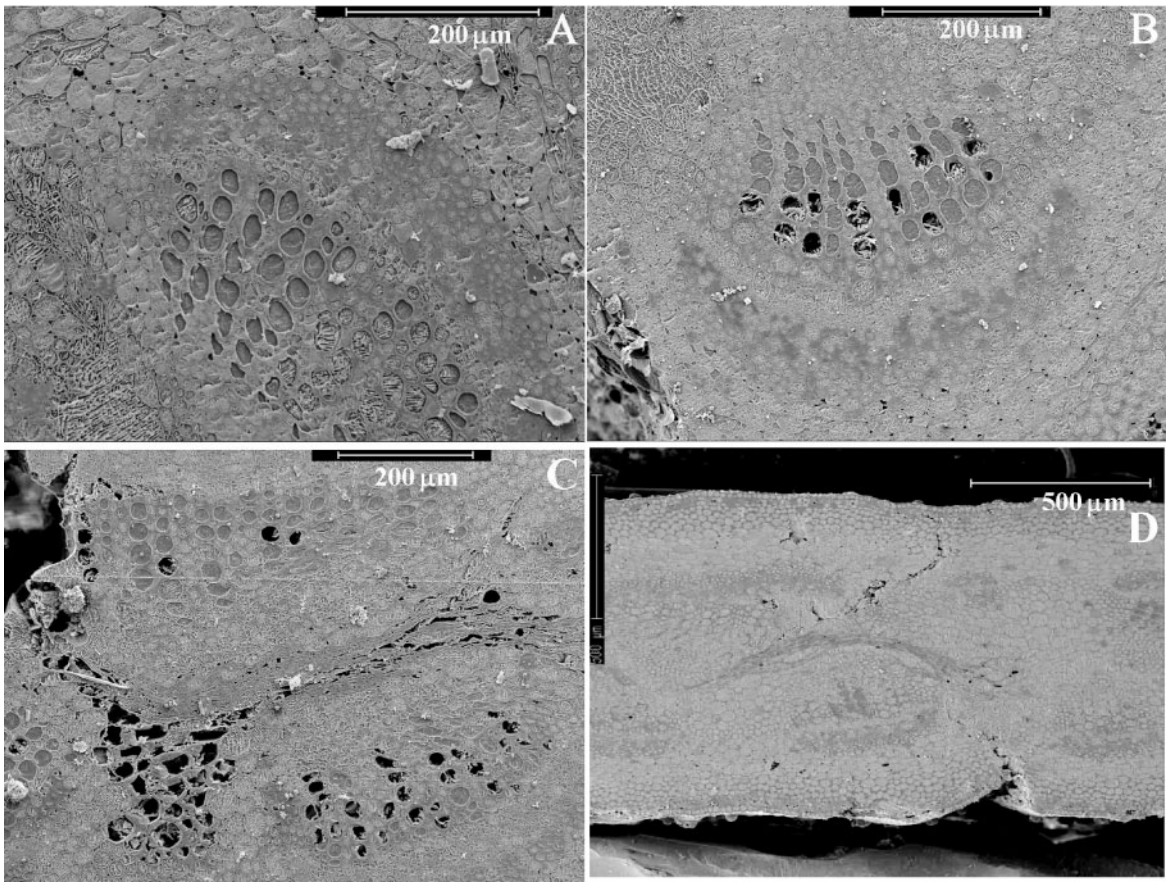

Fig. 9. Variation in the occurrence of gas-filled xylem vessels in petioles of cotton leaves during stomatal oscillations. (*A*) Petioles collected at 1244 hours showing almost all the vessels full of water (less than 1% were gas filled). (*B*) Petioles collected at 1305 hours, at this time 12.7% of the vessels were gas filled. (*C*) Petioles collected at 1321 hours when 17.9% of the vessels were gas filled. (*D*) Petioles collected at 1420 hours, when less than 1% of the vessels were gas filled. Percentage of embolism and collecting time correspond to data of Fig. 8.

to be the change in g per unit change in E that determines H, and not water stress *per se*. Therefore, treatments that increase root resistance, or hydraulic resistance generally, promote stomatal oscillations. These treatments include: stopping aeration (Barrs and Klepper 1968), cooling (Troughton 1969; Barrs 1971), root excision (Barrs 1971) and dissection of major veins (Beyschlag and Eckstein 2001). In fact, the transcription of several genes appears to be under circadian control in higher plants (Beator and Kloppstech 1996), including some transcripts involved in water transport in the root (Lopez *et al*. 2003). Further, the expression of some root aquaporin transcripts appears to be regulated by a circadian rhythm (Henzler *et al*. 1999; Clarkson *et al*. 2000).

The other component of total loop gain $\partial E / \partial g_s$ explains why oscillations occur in response to exposure of whole plants to high irradiance (Barrs 1971; Zipperlen and Press 1997) or increase in VPD (Cowan 1972; Farquhar and Cowan 1974). The system is more complicated when different environments are imposed on different leaves. We observed that the oscillations were damped when only one leaf was illuminated, and the rest of the plant was darkened. This presumably reflects an effective reduction in the loop gain when it becomes impossible to build up large changes in either g_s or E at the whole-plant level.

Effect of C_i and VPD on oscillations

It is well documented that, in many species, C_i influences stomatal aperture (Mott 1988). Further, as g increases, so too will C_i. So we have the makings of a feedback system where the physiological component is the dependence of g on C_i, and the environmental gain is $\partial C_i / \partial g$ (Farquhar 1973; Farquhar *et al*. 1978). Shorter-period oscillations have been associated with this CO_2-regulating system within the leaf (Barrs 1971). In our study, low C_i did not inhibit stomatal oscillations, and oscillations proceeded at the same intensity as observed under ambient CO_2 levels, confirming that the slower oscillations (period >30 min) are independent of C_i.

The amplitude of oscillation was greater at moderate VPD than at either low or high VPD. The observation is consistent with results reported by others (Cowan 1972; Wang *et al*. 2001). It may reflect two different tendencies. The first is for the environmental gain, $\partial E / \partial g$, to increase with increasing VPD. The second is for the mean level of g_s to decrease with increasing VPD, constraining transpiration.

Effect of oscillations on C_i

The value of C_i as normally calculated by gas exchange ($C_{i(gs)}$) is the g_s-weighted value (Farquhar 1989). At the troughs, the g_s-weighted C_i was greater than that estimated as a capacity-weighted value, obtained by extrapolation (i.e. $C_{i(CAP)}$ in Table 1; Fig. 5), probably as a consequence of non-uniform stomatal closure (Laisk *et al*. 1980; Terashima *et al*. 1988). Two observations support this hypothesis: First, gas-exchange data, from which the leaf area where stomata remained open (at the troughs) was calculated to be 39% (mean of troughs 1–8, Table 1), and second, imaging fluorescence data.

Parallel measurements of oscillations and fluorescence imaging permitted us to infer the relationship between F_m' and stomatal conductance. Stomatal patchiness, as determined by F_m', was more frequently observed at the trough phase of stomatal oscillations (Fig. 7), when patches of low F_m' values (light patches in Fig. 7) were observed coincident with gas exchange measurements of low g_s. This result is consistent with the report by Cardon *et al*. (1994), who observed an inverse relationship between non-photochemical quenching ($q_N = (F_m - F_m') / 0.8F_m$) and stomatal conductance. Beyschlag and Eckstein (2001) also observed maximum patchiness at the trough phase after inducing stomatal oscillations by interrupting the water flow in a major leaf vein.

Our study, one of a few to simultaneously monitor stomatal conductance and fluorescence parameters in different leaves from the same plant, supports the hypothesis that an 'overestimation' of C_i (as far as capacity is concerned) during the trough of stomatal oscillation is, at least in part, due to a heterogeneous decline in stomatal conductance, as evidenced from gas-exchange data (Table 1) or microscopic measurements of stomatal aperture (Laisk *et al*. 1980; Laisk 1983). Heterogeneity of stomatal conductance over the leaf surface implies, for example, that at very low g_s (i.e. troughs of Fig. 3) some stomata remain wide open whereas most are closed (Laisk *et al*. 1980; Laisk 1983). Our results also indicate that the maximum fluorescence yield (F_m') is a robust parameter for detecting stomatal patchiness. Similar results were obtained by Omasa and Takayama (2003) using non-photochemical quenching as an indicator of stomatal patchiness. West *et al*. (2005) also observed that fluorescence was related to stomatal conductance with non-dispersing patches of stomatal conductance regularly crossing a given region of a leaf, resulting in stomatal oscillations.

Synchronous oscillations

Stomatal oscillations have been observed to occur synchronously in all leaves of a single plant (Cox 1968; Farquhar 1973) and in fact only rarely have unsynchronised oscillations in stomatal conductance been reported (Teoh and Palmer 1971). In our experiments, synchronous cycling of stomata over the whole plant was inferred through simultaneous measurements of g_s and A in two leaves from the same plant (Fig. 6*A–C*). Reducing VPD in leaf 1 to ~0.5 kPa (at midday, Fig. 6*B*) did not stop oscillations, as should be expected if the whole plant were subjected to a decrease in VPD (Farquhar and Cowan 1974). However, a drop in VPD increased stomatal conductance, likely due to the effect of increasing humidity on stomatal aperture (Raschke 1979). VPD manipulation in leaf 1 clearly shows that oscillations were associated with the magnitude of the environmental gain at the whole-plant level, as leaf 1 oscillates in concert with leaf 2 exposed to a higher VPD (Fig. 6*C*). As VPD remained nearly constant during the day-time, it again appears plausible that oscillations faded away in the afternoon (Fig. 6*C*) as a result of decline in root resistance (i.e. decrease in H) rather than because of changes in $\partial E / \partial g_s$.

Xylem embolism

More xylem vessels were filled with gas at the peaks than at the troughs of stomatal oscillations. However, as the petioles were frozen while the xylem fluid was under tension, some doubts about artefacts remain. Thus, it is not possible to state unequivocally that there is a causal relationship between oscillations and embolisms. Nevertheless we raise the possibility that cavitation could be involved in the oscillatory process, without ruling out a potential artefactual effect of oscillations on cavitations.

In the latter case, an increase in transpiration as stomata open lowers leaf water potential and increases tension in xylem vessels, thereby increasing the probability that freezing causes cavitation near the peak of a stomatal oscillation.

On the other hand, although not proven, a causal link in the direction from cavitations to oscillations seems plausible. The water potentials that can trigger cavitation have been reported to be very close to those commonly experienced by actively transpiring plants in the field (Tyree and Sperry 1988; Nardini and Salleo 2000). Further, as noted earlier, the 20 min for refilling of embolised vessels in stems of *Laurus nobilis* (Salleo *et al*. 2004) is a time scale consistent with stomatal oscillations.

It is possible that as the xylem conduits in the petiole cavitate, g_s initially increases giving positive feedback before declining (Iwanoff 1928) and this may also increase the phase-shift between stomatal oscillations and xylem refilling (Fig. 8). Thus the wrong-way response would be

acting as a non-minimum phase element, as discussed by Farquhar (1973).

Conclusions

In this study we show the effect of whole-plant hydraulics on stomatal oscillations and stomatal patchiness. The importance of the hydraulic and environmental gains to oscillations is shown by comparing illuminated and non-illuminated plants. In non-illuminated plants, the hydraulic loop gain was too low outside the chamber to sustain oscillatory behaviour. Oscillations faded in the afternoon, probably as a result of a gradual decrease in root resistance, and causing a diminution of the gain of the hydraulic transfer function (reduction in H). In some stomatal oscillations, stomatal patchiness was observed at the trough phase. In the troughs some stomata remain open, and it is for this reason that some photosynthesis was still observed in the troughs at night, in illuminated leaves. Reducing C_i did not suppress oscillations, showing that oscillations reported in this study were caused by the whole-plant hydraulic system. If cavitation is involved in oscillations, the delays in refilling of cavitated vessels may also contribute to the phase lag in H, the internal physiological component of the dynamics, which leads us to suggest that the dynamics of the plant hydraulic system may be much more important than previously recognised. However, as noted earlier, possible limitations of the cryo-SEM technique make us cautious in attributing an effective role of cavitation in oscillations. Nevertheless, our results are obviously suggestive, and so further experiments are needed to test this possibility.

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