

Circadian Rhythmicity in Excised *Samanea Pulvini*

II. RESETTING THE CLOCK BY PHYTOCHROME CONVERSION¹

Received for publication March 8, 1976 and in revised form June 4, 1976

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ABSTRACT

Excised *Samanea saman* pulvini were incubated in H₂O or 50 mM sucrose in darkness for 100 to 152 hours except for brief exposures to red or far red light, and angles of opening measured periodically. When pulvini are incubated in H₂O, the rhythm damps in the open position after two to three cycles irrespective of the light treatments, but when sucrose is available, the now persistent oscillations show large red, far red-regulated effects on phase, amplitude, mesor slope, and entrainment. Single red light pulses rephase the rhythm, with a phase response curve that resembles that reported for other plants and animals; such rephasing is prevented by immediately subsequent far red light, indicating that phytochrome is the photoreceptor. Red light pulses repeated every 24 hours entrain the rhythm, and also prevent damping if presented at an appropriate part of the cycle.

The rhythmic movement of intact *Samanea* leaflets or of their excised pulvini is affected both by prolonged, high intensity white light (8, 13, 18) and by brief, low irradiances with R³ or FR absorbed by phytochrome (14, 20). Previous investigations on phytochrome photoconversion all involved administration of light during the first 24 hr of darkness following the usual light-dark regimes of plant growth chambers. They could not, therefore, reveal whether phytochrome affected rhythmic entrainment, as would be expected if it acted on the clock itself, or whether it simply modified the coupling of the time signal to the motor mechanisms in the pulvinus. To answer this question, it is necessary to probe a greatly extended dark period with R and FR light signals given at various times. The present investigations, describing such experiments with excised *Samanea* pulvini, provide data essential for an analysis of light-rhythm interaction.

MATERIALS AND METHODS

Samanea saman plants were grown from seed under 16-hr light-8-hr dark cycles in controlled condition growth chambers as previously described (13). Secondary pulvini attached to a small section of rachilla were excised, laminar tissue removed, cut rachillar ends supplied with H₂O or sucrose, and angles measured as described in reference 13. One member of a pair of

pulvini received R and the other FR in all experiments comparing these two light treatments. Light sources for phytochrome photoconversion (R = 3- or 5-min exposure at 2.2 J m⁻², 600 to 690 nm, and FR = 1.5-min exposure at 9 J m⁻², 710 to 750 nm) and the dim green light used during the measurements and experimental manipulations are described in reference 15. The beginning of the dark period, DD = 0, coincides with the regular lights off signal.

RESULTS

Excised pulvini incubated in H₂O during DD oscillate for only one cycle before the rhythm damps in the open position (18). R or FR administered at regular intervals (Fig. 1) permit the rhythm to persist for two to three cycles, although the amplitude of the oscillations and R-FR differences tend to decrease with time, and oscillations cease after 72 hr darkness. Since sucrose is required for sustained rhythmicity in DD (18), all pulvini were supplied with 50 mM sucrose in the remaining experiments. As will become apparent (see Figs. 5 and 6), R and FR have very different effects on the phase, period, and amplitude of the movement, when sucrose is available.

Two types of experiments were performed. To investigate phase response, one R or FR light pulse was presented at different times of the rhythm. To test for rhythmic entrainment, pulses of R or FR light were presented every 24 hr. In all cases, the effects of light treatments were compared to free running dark controls.

Phase Shifting with One Red Light Flash. The effects of R presented at 25 different times during a long dark cycle were analyzed; Figure 2 indicates the results of eight such treatments, while Figure 3 compares the effects of all 25 treatments. One R pulse presented during the second two-thirds of pulvinal opening or the first half of closure reduces the duration of the next cycle, thereby producing a phase advance, while one pulse presented during the second half of pulvinal closure, or the first third of the opening movement increases the duration of the next cycle, thereby causing a phase delay. R presented at the middle of closure has no effect on the phase; we have designated this as the ZPST.

Phase and Amplitude Response Curves. The amount of phase shift was determined by comparing the time of the first maximum after the R light treatment with that of the same maximum in the dark control. R produces a maximum phase shift of about 12 hr when presented during pulvinal opening, 12 hr from ZPST. The amount of phase shift depends primarily upon the interval between the light pulse and ZPST and is remarkably independent of the cycle in which the R treatment is presented, except for the first 8 hr of DD. R at this time is much less effective than if presented at an equivalent part of a later cycle, as also reported in other plants (5, 22). R light pulses that rephase the rhythm also change the amplitude of the oscillation

¹ This work was supported by a grant from the Spanish Ministerio de Educación y Ciencia to E. S. and by National Science Foundation Grant BMS74-24269 to R. L. S. and A. W. G.

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³ Abbreviations: DD: prolonged darkness interrupted by (R) brief red or (FR) far red as indicated; ZPST: zero phase shift time, *i.e.* the time when light has no effect on the phase.

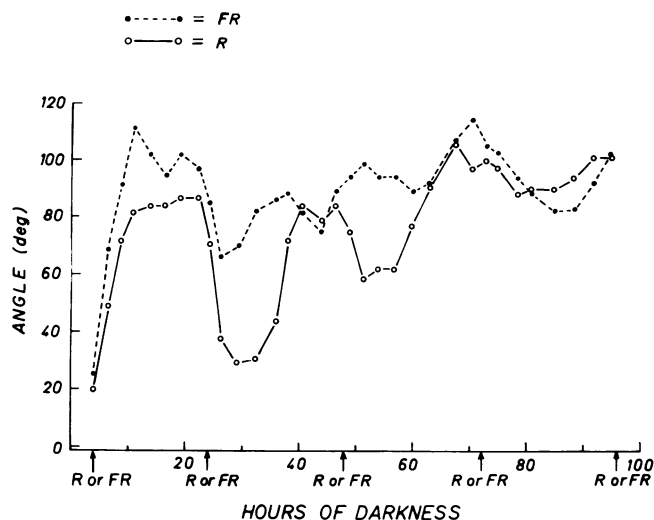


FIG. 1. Oscillation in the angle of *Samanea* pulvini attached to a small section of rachilla. Pulvini were excised at DD = 2.5 hr and the cut end of the rachilla immersed in H₂O. They were irradiated with R (3 min) or FR (1.5 min) at DD = 4, 24, and every 24 hr thereafter.

and the slope of the mesor (median angle).

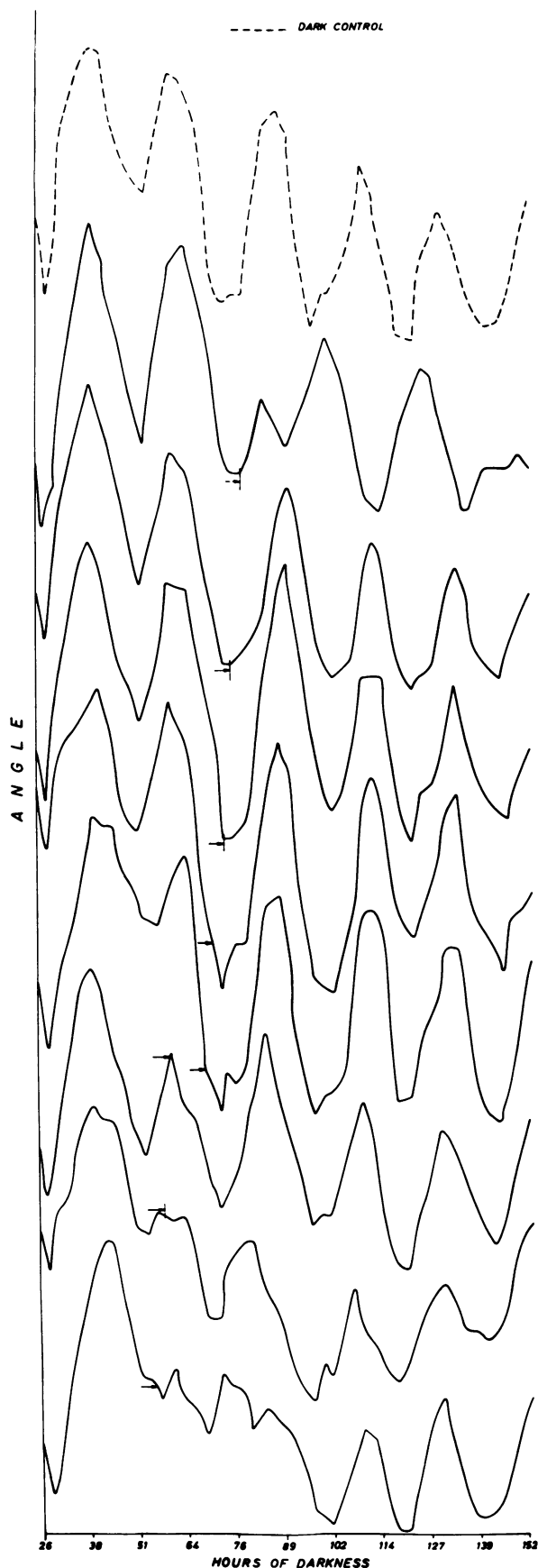
Measurements of phase shift and other associated changes were not continued for more than three cycles after R, due to senescence of the excised pulvini. It is difficult to draw firm conclusions about the stability of these changes, although data in Figure 2 suggest that phase delays are more stable than advances. R treatments during the second cycle seem to provide the most stable effects.

FR Reversibility of Effects Potentiated by R. To determine whether the effects of R on rhythmic phase and amplitude are reversible by FR, pulvini were exposed briefly to the following four sequences: R; FR; R, FR; or FR, R. In some experiments, R was presented at times that elicit a phase advance, while in the other experiments, R was timed to elicit a phase delay. In all cases, the behavior of FR and R, FR-treated pulvini mimicked that of dark controls, while FR, R (Fig. 4) was as effective as R in altering the phase and amplitude of the rhythm. These experiments leave no doubt that red light effects on phasing are mediated by phytochrome.

Rhythmic Entrainment by Repetitive R Pulses. Figures 5 and 6 compare the effects of R and FR presented at 24-hr intervals. FR-irradiated pulvini have the same phase and period as the dark control no matter when they are irradiated, although they show small differences in amplitude and mesor slope if the first light treatment is given at hr 4. However, R-irradiated pulvini differ significantly from the dark control in phase, amplitude, and mesor slope, no matter when the light treatments are given. It is clear that R pulses entrain the rhythm.

The number of cycles necessary for complete entrainment depends upon the interval between the first R treatment and ZPST. When pulvini are irradiated a few hr before or after ZPST, one or two cycles are sufficient, but when the light treatments are presented 10 to 12 hr from ZPST, more than four cycles are required. In all cases, if the light treatments are given at 24-hr intervals, they coincide with ZPST after entrainment. Some light schedules (e.g. upper R-irradiated curve, Fig. 5)

FIG. 2. Oscillation in the angle of excised pulvini supplied with sucrose and irradiated with R (5 min) at the times indicated by arrows. Curves are arbitrarily arranged on the ordinate for convenient viewing; no numerical differences in amplitude are implied between curves.



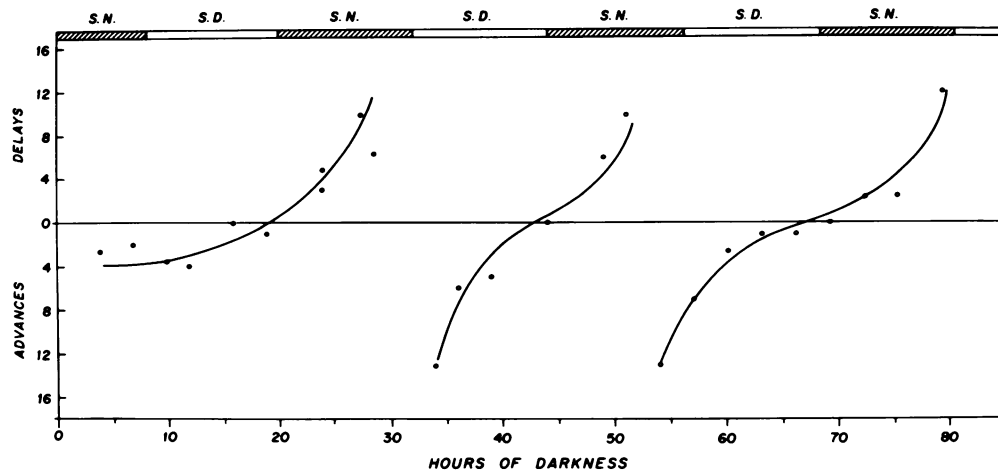


FIG. 3. Phase response curve showing the phase shifting effect of a single 5-min red light pulse. Abscissa: time of R treatment; circles: phase advance or delay compared to a dark control; solid lines: best fitting curves for each cycle.

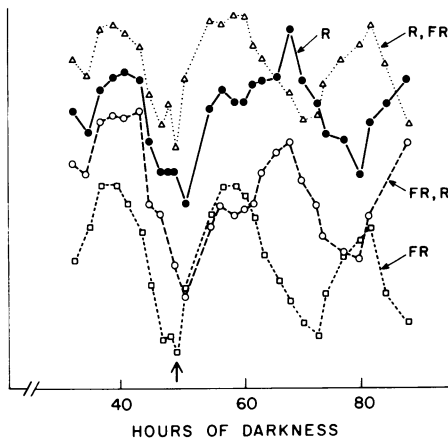


FIG. 4. FR reversibility of the phase shifting effect of a single R light pulse. Pulvini were irradiated at DD = 48 with R (5 min), FR (1.5 min), R followed by FR, or FR followed by R. The ordinate indicates angle; however, curves are arbitrarily arranged for convenient viewing and no numerical differences in amplitude are implied between the curves.

prevent the progressive decrease in the amplitude of the oscillations that characterizes the behavior of dark controls.

DISCUSSION

It is clear that phytochrome is a photoreceptor for rhythmic phasing and entrainment in *Samanea*, as also reported in *Bryophyllum fedtschenkoi* (21), *Lemna perpusilla* (5), *Phaseolus multiflorus* (1), and *Kalanchoë blossfeldiana* (16, 22), although Halaban (4) concluded that phytochrome is not involved in the leaf movement rhythm in *Coleus*. Pigments other than phytochrome regulate phasing in *Neurospora crassa* (11), *Gonyaulax polyedra* (19), and *Drosophila pseudoobscura* (9). The phase response curve reported in this paper for *Samanea* resembles those reported for other organisms, irrespective of the pigment involved in rhythmic rephasing. Thus, a unifying concept to explain light-clock interaction must necessarily be based on events that can be regulated by a variety of pigments.

The *Samanea* system has certain advantages for analyzing light action on the clock. In some other plants with rhythms entrained by Pfr, long irradiation periods are required (16), but in *Samanea*, very brief red light pulses suffice; this is advantageous for identifying early, photoregulated events that affect the clock. These early events might involve control of ion movements. In *Samanea* and *Albizzia*, K^+ (13-15), Cl^- (17), and transmembrane potential (10) are regulated by phytochrome and high intensity white light, the latter possibly acting via the same blue absorbing flavoprotein that appears to regulate rhythmic phasing in *Neurospora* (7, 11). A blue absorbing pigment regulates leaflet opening in *Albizzia* (2, 6) and probably in *Samanea* (3). It will be of interest to determine whether blue light also affects rhythmic entrainment in *Samanea*, and if so, whether it is most effective if presented during leaflet opening.

Data in Figure 5 reveal that oscillations persist without damping for 150 hr DD when pulvini are exposed to R at an appropriate part of each rhythmic cycle. Relatively little K^+ is lost to the bathing solution during this time (18), supporting the view expressed elsewhere (12, 17) that K^+ and Cl^- might migrate in a closed system, such as cell-to-cell movements through plasmodesmata in the symplast.

Implications Regarding Pfr Stability. Irradiation during the photoperiod is provided by cool white fluorescent lamps (about 2000 ft-c with about 10% incandescent supplement) this source should convert a high per cent of the phytochrome to the Pfr form. Thus, the ineffectiveness of R given during the first 8 hr in eliciting a phase shift is probably due to a high Pfr level resulting from the previous white light period. Previous data (14) also suggest that Pfr is relatively stable during the first 8 hr of DD; however, it is clear that the Pfr level decreases during longer dark periods, since dark controls and FR-irradiated pulvini behave similarly (Fig. 6).

Sucrose and Pfr Requirements for Opening and Closure. When dark controls are incubated in H_2O , the rhythm damps completely in an open position after one to two cycles. Although oscillations persist for several cycles when sucrose is available, the mesor decreases progressively. The only treatment that prevents such damping is daily R irradiation, at appropriate times. Thus, in *Samanea*, as in *Albizzia* (12), both sucrose and the conversion of Pr to Pfr at a particular part of the rhythmic cycle are required for sustained oscillations of maximum amplitude.

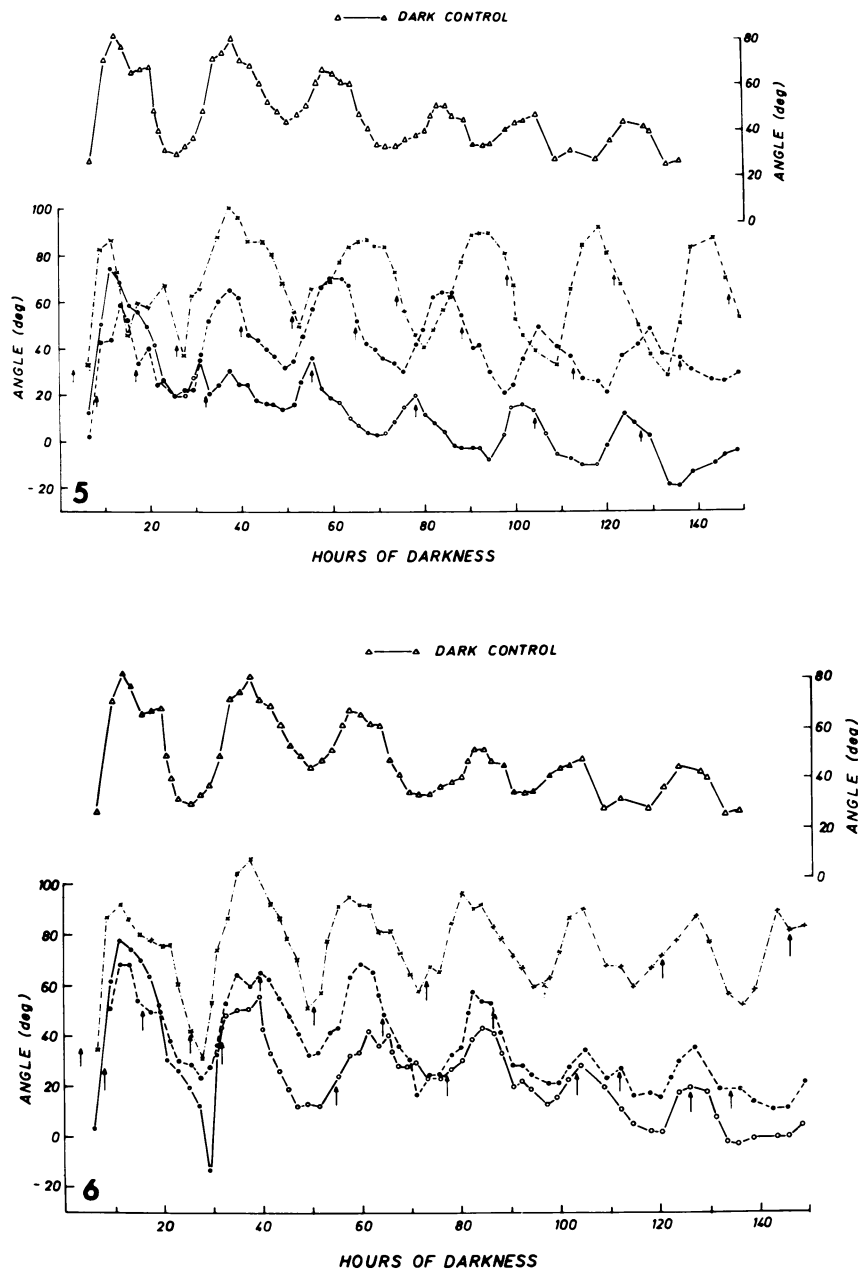


FIG. 5, 6. Oscillation in the angle of excised pulvini supplied with 50 mM sucrose, and exposed to R (3 min) (Fig. 5) or FR (1.5 min) (Fig. 6) at 24-hr intervals, as indicated by arrows.

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