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Quantifying Rhythmic Movements of *Albizzia julibrissin* Pinnules[1]

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WILLARD L. KOUKKARI
Department of Botany, University of Minnesota, Minneapolis, Minnesota 55455

FRANZ HALBERG
Department of Laboratory Medicine and Pathology, Chronobiology Laboratories, University of Minnesota, Minneapolis, Minnesota 55455

SOLON A. GORDON
Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Illinois 60439

ABSTRACT

The cosinor technique, previously applied to studies of animal rhythms, is used to assess the circadian rhythm in pinnule movement of *Albizzia julibrissin*. The method provides a quantitative approach for studying rhythm properties of either intact or excised pinnules. Phase shifting in *A. julibrissin*, as compared to the usually slower phase shifts of various circadian phenomena in the insect, bird, and mammal, occurs within 1 day or two. Rhythm adjustment in the pinnules takes place more rapidly when the lighting regiment is advanced than when it is delayed.

Leaf movement rhythms have long been studied by exclusive reliance upon inspection of data recorded in time plots (1, 3). In the meantime, progress also has been made toward quantifying rhythms by objective methods which included inferential statistical techniques for interpreting the data (4, 10). In this communication, serial cosinors (7) are introduced into the analysis of circadian pinnule rhythms, to focus especially on their phase shifting following advances and delays in lighting schedule. The extent of precision and reproducibility of this objective technique as applied to a plant rhythm, and differences in phase shift behavior thus assessed, are the two major points presented.

MATERIALS AND METHODS

Albizzia julibrissin plants were propagated from seeds and kept in the greenhouse for spans of varying lengths (weeks to months); however, to be included for study, a plant had to have two or more mature leaves. Plants were transferred to growth chambers at least 3 days before each study and were maintained at 24 ± 2 C on a regimen of 16 hr of light (supplied by cool white fluorescent lamps and two or four 40- or 60-w incandescent lamps (about 1000–2000 ft-c)) alternating with 8 hr of darkness.

The angle formed by a pair of pinnules was measured to the nearest 10° by comparing it with a set of model angles, each cut as a paper or plastic triangle (10°–180°). The relative locations of the pinnules, pinnae, and leaves have been identified and described in previous studies (9). A green safelight was used when measuring angles during D.[2] The safelight consists of two 15-w daylight fluorescent lamps shielded first by a sheet of amber Plexiglas (Rohm & Haas, No. 2422) one-eighth inch thick, followed by a similar size piece of blue Plexiglas (No. 2424).

Pinnule angle measurements at 1- to 3-hr intervals were coded onto punch cards for computer analysis by least squares. A 24-hr cosine curve was fitted to the data to obtain point and interval estimates of three parameters, namely the mesor, amplitude, and acrophase—approximations of the data shown in Figure 1.

The mesor corresponds to the best fitting (rhythm-adjusted) average value and may be illustrated by a line bisecting the fitted cosine curve half-way between its peak and trough. The amplitude represents the distance from M to the peak of the cosine function approximating the rhythm. The acrophase locates in time the peak of the curve best fitting the data.

Since circadian denotes periods varying from 20 to 28 hr, including the precise 24-hr period, and since a 24-hr cosine was consistently fitted for the analyses presented, all ϕ values are circadian ϕ values. Such a ϕ can be given as the lag of the cosine's peak from the zero phase, chosen here as midnight. The lag is expressed in hours and minutes or in degrees: since 24 hr are equivalent to 360°, 1 hr equals 15°.

The above mentioned parameters A, M, and ϕ from the fit of a single cosine curve were used for quantifying pinnule movement rhythms, without estimating any nonsinusoidality of the waveform by fitting added harmonics, or by other approaches which have been adequately discussed and illustrated elsewhere (16). The cosine curve and the calculated M, A, and ϕ describe objectively the prominent sinusoidal features of a

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[2] Abbreviations: D: dark; M: mesor; A: amplitude; ϕ: acrophase; L: Light.

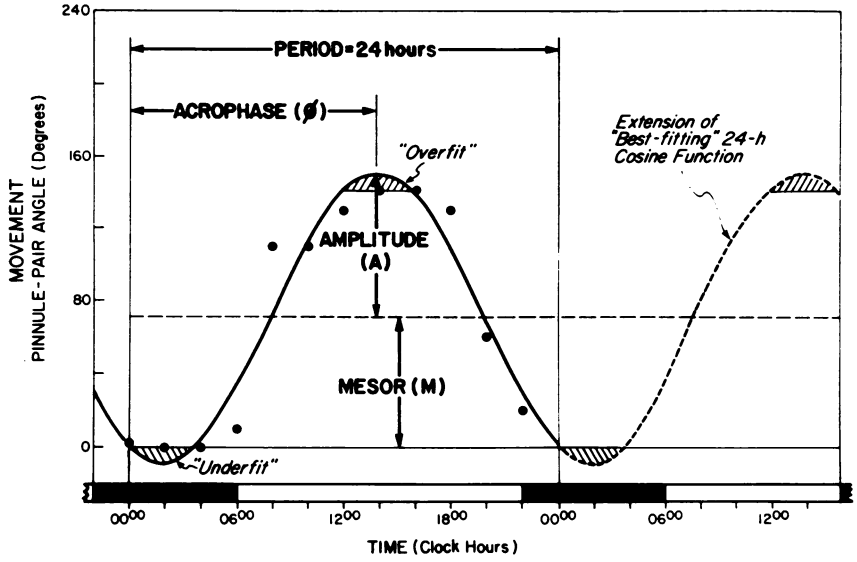

FIG. 1. Characteristics of pinnule pair movement rhythm of *A. julibrissin* obtained by the fit of a single 24-hr cosine curve. Angle for pinnule pair No. 5 measured at 2-hourly intervals on pinna A', A, B', and B of leaf No. 2. Each dot is the mean of 4 angles. L:0600 to 2200 hr; D:2200 to 0600 hr.

rhythm—even when data are scarce or available at unequal intervals. While the data in some of our experiments are consistently collected at identical intervals (Fig. 1), experimental conditions often are such that measurements cannot or need not be collected at equal or frequent intervals. In these instances the procedures described in this paper are particularly useful.

It should be noted that a continuous record, or at least a large number of measurements obtained at regular intervals, was usually required not only to describe a rhythm solely from the examination of a time plot displaying original values but even for some early analytic techniques that required dense as well as equidistant data. By contrast, the fit of a cosine curve(s) requires fewer observations for quantitative interpretation, as long as there is some approximation of sinusoidality in the data.

When measurements are obtained at irregular time intervals and the fitted cosine curve properly approximates the rhythm in the data, M is more representative of the true 24-hr average than is the arithmetic mean. The mean is greatly influenced by extreme values when measurements are obtained, for example, at arbitrarily chosen times which might well coincide with spans of either maximal opening or maximal closing of the pinnules.

Few systems of analysis are perfect. The curve-fitting method we used should be qualified in several ways. First, most biologic waveforms remain unevaluated or incompletely described by the fit of a single cosine. This circumstance can obscure differences that are apparent to the naked eye. For instance, the rhythm parameters M, A, and ϕ, may be similar for rapidly and slowly opening pinnules. The ϕ evaluates only the "center of gravity" in the location of the high values which can be the same for two groups being compared, even though pinnule pairs *in vitro* and *in vivo* may open with different speed.

Second, even when judiciously used, the fit of a cosine may lead to artifacts such as the values displayed below the zero line in Figure 1. This slight undershoot occurs during D when pinnule angles are recorded as zero; hence A appears to be larger than M (Fig. 1). This situation can be compared to a number of biochemical studies where analyses indicate a recovery of over 100% of the standard (5). In this same connection, it is pertinent that when one pinnule from a pair is removed, the tip of the remaining pinnule moves across the rachilla: one could hypothesize that a negative value for the angle would have been obtained had the two pinnules (of a pair left in place) not blocked each other from moving beyond the zero position. It is also noteworthy (although not presented here) that movements of the isolated pinnules resulting in negative angles occur very early in D and persist in that position for hours. However, Figure 1 shows as hatched areas the "overfit" of the cosine curve, in regions where this curve exceeds the highest and drops below the lowest value.

RESULTS AND DISCUSSION

Results of the experiments are summarized as circadian parameters in Tables I and II. The point estimates of the circadian acrophases of the pinnule movement rhythm for the two plants, with their confidence intervals, lie between −184° (−169° to −199°) and −204° (−188° to −219°). The point estimates of the mesor lie between 50 ± 11 and 83 ± 6, and those of amplitude lie between 62 ± 9 and 85 ± 18. The interplant difference in acrophase (Table I) emphasizes the desirability in physiological studies on nyctinasty of using corresponding pinnule pairs from opposite pinnae (*e.g.*, pinnule pair No. 4 from pinna A and No. 4 from pinna A') to obtain materials which are comparable both physiologically and morphologically (9).

Any effects of excision on rhythms in pinnule pair movements (Table II) are not evident in our analyses. In fact, good agreement between rhythm parameters of intact and excised pinnules is observed. Point estimates in Table II for A differ by only 9° and for ϕ by less than 1 hr.

A subsequent study is summarized by the cosinor display in Figure 2 of data from a plant on an LD 16:8 regimen. More specifically, the rhythm characteristics (A, ϕ) of separate series, each representing the movements of pinnule pairs from different pinnae, can be represented in the form of a polar plot (the cosinor, Fig. 2). The mean value of ϕ is expressed as an angle, with the period of the rhythm being equated to 360° (= 24 hr) and A being represented by the length of a directed line (by a vector). The oval at the end of the vector depicts the 95%

Table I. *Extent of Agreement among the Circadian Parameters, Mesor, Amplitude, and Acrophase*
Results are calculated for movement rhythm of individual pinnule pairs on the same or different (I *versus* II) plants.

Pinnule	Pinnule Pair	Mesor ± SE	Amplitude ± SE	Percentage of Rhythm[1]	Acrophase (0.95 confidence arc)
Experiment I[2]					
A	4	70 ± 5	75 ± 7	91	−190° (−180° to −201°)
	6	71 ± 5	76 ± 7	92	−189° (−179° to −199°)
	8	66 ± 6	68 ± 7	88	−193° (−180° to −206°)
	10	66 ± 6	68 ± 7	88	−193° (−180° to −206°)
A′	4	69 ± 6	80 ± 8	92	−185° (−175° to −196°)
	6	64 ± 6	77 ± 7	91	−188° (−177° to −199°)
	8	62 ± 4	77 ± 6	94	−189° (−180° to −198°)
	10	63 ± 5	77 ± 7	92	−188° (−178° to −198°)
B	4	50 ± 11	85 ± 18	74	−197° (−177° to −218°)
	6	57 ± 6	71 ± 8	88	−190° (−177° to −203°)
	8	59 ± 6	70 ± 8	87	−188° (−174° to −201°)
	10	57 ± 6	62 ± 9	83	−184° (−169° to −199°)
B′	4	58 ± 5	79 ± 7	92	−189° (−179° to −200°)
	6	60 ± 5	73 ± 7	91	−188° (−177° to −199°)
	8	59 ± 6	70 ± 8	87	−189° (−176° to −202°)
Experiment II[2]	10	54 ± 5	65 ± 6	90	−190° (−179° to −201°)
A	4	77 ± 6	73 ± 7	76	−204° (−189° to −218°)
	5	78 ± 6	70 ± 8	71	−203° (−187° to −219°)
A′	4	83 ± 6	74 ± 8	73	−204° (−188° to −219°)
	5	83 ± 6	73 ± 8	72	−204° (−188° to −219°)

[1] Percentage of variability accounted for by fitted model (here a 24-hr cosine model).
[2] In light from 0600 to 2200 hr alternating with darkness for 3 days prior to start of study. In experiment I, pinnules 3, 5, 7, 9, 11, and 13 were removed from the rachilla.

Table II. *Extent of Agreement among Circadian Amplitudes and Acrophases Computed for Intact and Excised Pinnule Pairs of A. julibrissin*

Source of Pinnule Pairs	Amplitude[1] ± SE	Acrophase	Limits (0.95 confidence arc)
Intact (on the plant)			
Exp. I, Table I	73 ± 3.8	−190°	−188° to −193°
Exp. II, Table I	73 ± 3.1	−203°	−201° to −205°
Excised (in petri dish containing water)			
Exp. III[1]	64 ± 9.7	−196°	−174° to 209°

[1] For each experiment, hypothesis of zero amplitude rejected at below 0.2% level.
[2] Five or six excised pinnule pairs per dish, totaling 21 excised pairs.

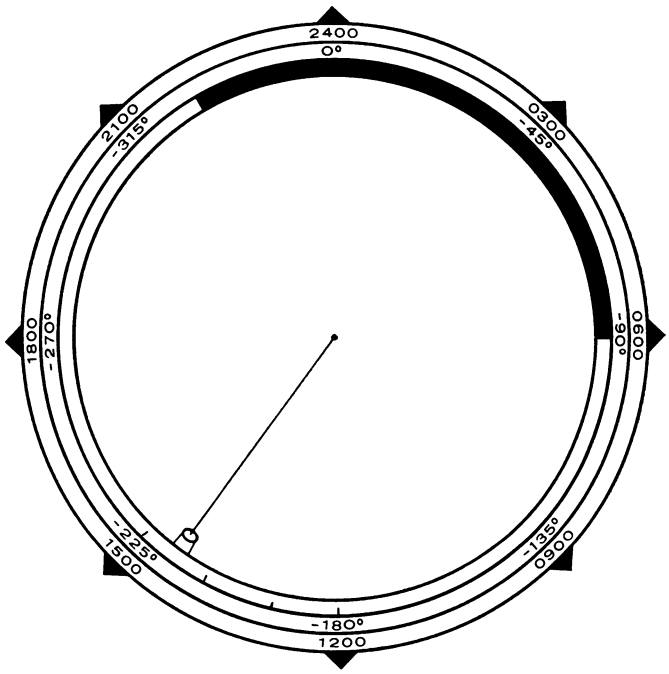

FIG. 2. Cosinor of *A. julibrissin* pinnule movement rhythm. Amplitude (length of vector, A) equals 85° (as change in pinnule angle) and has a 0.95 confidence interval of 83° to 86°. Acrophase (angle of vector, ϕ) at −217° has a 0.95 confidence interval of −215° to −219°. Eight replications from eight different pinnae A′A–D′D, all from leaf No. 1, each represent the mean of movements by pinnule pairs 5, 6, and 7 (8). L:0600 to 2200 hr; D:2200 to 0600 hr.

confidence region for A and ϕ. Figure 2 reveals a tight oval, hence the characteristic group rhythm is well approximated. Had the replications yielded less similar results, the oval confidence region would have been larger; had (A, ϕ) results on individual series been grossly dissimilar, the oval would have overlapped the pole (the center of the plot) and a characteristic group rhythm (if it occurred) would not have been detected.

In continuous darkness and in continuous light, the rhythm persisted for at least 4 days. Cosinor summaries on these data —beyond our scope herein and hence not shown—demonstrate a highly significant group rhythm of *A. julibrissin* pinnule movements in both DD and LL: neither any inter-pinnule pair desynchronization in DD or LL nor the change in lighting regimen, as such, obscure the rhythm within the span investigated.

In a number of physiologic studies, including phytochrome effects of nyctinasty (9, 11, 12, 14, 15), it is essential to know how long a time span is required for the pinnules to adjust to a new timing of the lighting schedule. The results of three sepa-

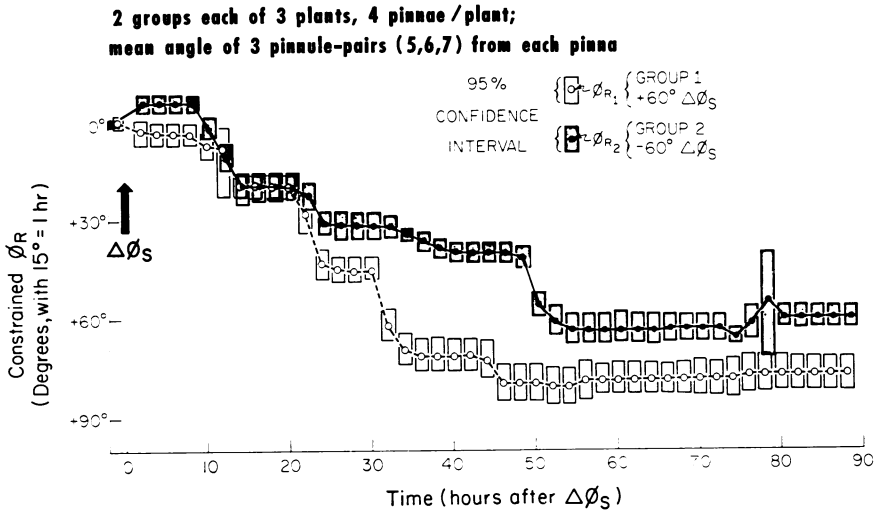

FIG. 3. Circadian acrophase, ϕ_R, for pinnule movement of *A. julibrissin* showing a more rapid adjustment following the 4-hr shortening of a single light span, a synchronizer advance (□) than after its lengthening by a delay of 4 hr (■).

rate experiments indicate that phase shifting occurs rapidly in *A. julibrissin*. A single span of darkness was shortened or lengthened by 4 hr to obtain advance or delay of rhythm, respectively. One of these studies is illustrated by the experiment described in Figure 3. The shift in the rhythm's acrophase, ϕ_R, is computed by fitting a 24-hr cosine curve to 24-hr sections of the entire series, these intervals being displaced in 2-hr increments (6). An intergroup difference in ϕ_R shortly after the outset may be due to a pre-ϕ_S difference, a point that cannot be resolved in view of the brevity (3 days) of initial observations.

In any event, when the LD 16:8 lighting regimen is advanced by 4 hr ($\Delta\phi_S = +60°$), the shift of the movement rhythm occurs more rapidly than after a delay of 4 hr in the lighting regimen.

More specifically, the initial fit of a cosine curve (as a first step, *e.g.*, Fig. 1) and the series of cosinor analyses in Figure 3 provide an objective method for the inferential statistical study of rhythm characteristics in plants. As illustrated by the phase shift study of pinnule movements in Figure 3, one also is able by such techniques to quantitatively compare rhythm characteristics—for instance, following changes in schedule. Thereby one detects and quantifies differences in rate of phase shift as a result of advances or delays in the lighting regimen. Indeed, when the confidence intervals of the circadian ϕ values for groups 1 and 2 in Figure 3 do not overlap, they are rather similar during the first 30 hr after the change in lighting regimen (ϕ_S); however, between hour 30 and hour 50 thereafter they differ greatly, a finding indicating a difference in shift time of the rhythm. Moreover, during the remainder of the observation span (after hour 50 following [$\Delta\phi_S$]), there are again only small differences between ϕ_{R1} and ϕ_{R2}; they probably can be explained by differences at zero phase between the two groups. These differences might be eliminated by having a longer initial observation span than the 3-day records obtained before the change of lighting regimen. In any event, the differences in shift time shown in Figure 3 have precedents in many other life forms.

Also of comparative physiologic interest are the results reported earlier for other organisms, variables, and approaches (1, 2, 7, 13), notably those that show convincingly that, following manipulation of the lighting regimen, a shift of rhythm in one direction usually occurs much more quickly than an adjustment in the opposite direction. Such a polarity of directedness in phase adjustment (7) probably is related to the stage of the circadian system when the phase shifting stimulus is applied. The circadian system phase indeed critically determines the outcome of adjustment subsequent to stimulation in many cases, as documented by drastic differences in response not only to light but also to a variety of agents—noise, drugs, bacterial endotoxins, to cite but a few examples (1, 5).

As a comment on the techniques here introduced, biologic rhythms are not necessarily sinusoidal; special waveforms require special techniques of analysis (8, 16). However, the fit of cosine curves represents a rather generally useful approach whether or not the waveform is sinusoidal. One must keep in mind that we use microscopes to resolve cells, without assuming that the microscope constitutes a model for the cell. By the same token, one can fit cosines to data on biologic rhythms without assuming that the cosine constitutes the model for a given rhythm.

For the specific case of the pinnule movement rhythm, it is important to realize that the cosine fit does account for a sizeable percentage of the over-all variability. Table I shows that the index "per cent rhythm" or the percentage of total variability accounted for by the fitted cosine curve ranged from 71 to 94%.

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