Computer Analysis of the Leaf Movements of Pinto Beans[1]

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Abstract. Computer analysis was used for the detection of rhythmic components and the estimation of period length in leaf movement records. The results of this study indicated that spectral analysis can be profitably used to determine rhythmic components in leaf movements.

In Pinto bean plants (*Phaseolus vulgaris* L.) grown for 28 days under continuous light of 750 ft-c and at a constant temperature of 28°, there was only 1 highly significant rhythmic component in the leaf movements. The period of this rhythm was 27.3 hr. In plants grown at 20°, there were 2 highly significant rhythmic components: 1 of 13.8 hr and a much stronger 1 of 27.3 hr. At 15°, the highly significant rhythmic components were also 27.3 and 13.8 hr in length but were of equal intensity. Random movements less than 9 hr in length became very pronounced at this temperature. At 10°, no significant rhythm was found in the leaf movements. At 5°, the leaf movements ceased within 1 day.

Leaf movements have been extensively used to study the phenomenon of circadian rhythms. It has been hypothesized that the leaf movements indicate the presence and also the phase of the circadian rhythm in the plant. Various light treatments, temperature regimes and metabolic inhibitors have been given to plants to test for changes in this rhythm (4, 5, 6). In some cases the interpretation of data has been difficult because the leaf records generally contain short period movements which may obscure changes in the circadian rhythm.

In an analysis of rhythmic movements, the period of the movements is usually determined by the time interval between consecutive peaks or between consecutive dips in the recorded movements. However, as mentioned previously, it is difficult in some records to determine by visual measurement precisely when the peaks or dips occur. Without careful and extensive analysis of this type of data, it is possible to make erroneous conclusions. Because of this weakness a procedure is presented for the analysis of leaf movement data with the aid of a computer. Arbitrary evaluations of data are minimized by this technique and the extensive computations which are required for a thorough analysis of such data can be performed. It was hoped that by spectral analysis, it might be possible to determine accurately the

period of the circadian rhythm and also to determine other rhythmic components which may be present in leaf movement records.

Materials and Methods

Multiple Time Series Spectral Estimation programs, BMDX73 and BMDO2T, developed by the Health Sciences Computing Facility of U.C.L.A. were used (2). A newer program, BMDX92, which computes power spectra several times faster than these programs was used in the most recent analysis. These programs estimate the auto-spectra, cross-spectra and coherences for stationary time series. It is recognized that leaf movement records do not constitute a true stationary time series since the record does not repeat as would data from an oscillating physical-mechanical object. However, it is possible to estimate the length and the power of the periods in leaf movement records with these programs. The mathematical steps involved and the procedures used in spectral analysis will not be discussed here. Only information necessary to interpret the graphs and results will be presented. Readers are referred especially to Blackman and Tukey (1) and to Walter (10) for further information on spectral analysis.

A spectral estimate of data containing only a 24.0 hr periodicity and no other will be a sharp peak (Fig. 1), whereas a spectral estimate of data containing periodicities ranging from 20 to 28 hr with a mean of 24 hr will be a broad peak extending from 20 to 28 hr centered at the 24-hr point. If with values above zero a definite peak is not present, such values may be considered "noise". Factors
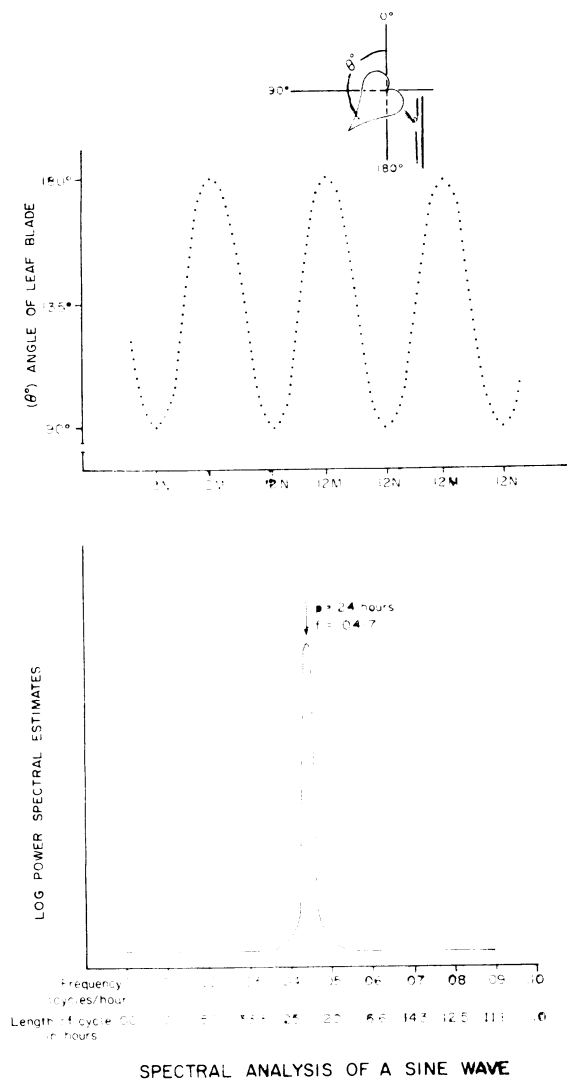
SPECTRAL ANALYSIS OF A SINE WAVE

FIG. 1. Leaf angle measurements and spectral estimates. Hypothetical leaf movement data (sine wave) with peaks occurring 24.0 hr apart are plotted in the upper graph. The lower graph depicts the power spectrum of the hypothetical data in the upper graph. Values assigned to the leaf angles are depicted at the upper right. In the lower graph, the abscissa is presented as frequency (f) in cycles per hr (cph). The second scale for the abscissa is expressed in the corresponding values of period length (p) in hr (h). The ordinate is expressed as the logarithm of the power.

such as measurement errors, time of watering, air movements and nutation movements will contribute to the "noise" value. On the other hand, the presence of a distinct peak indicates a preponderance or power of that particular frequency. If the power of this peak is significantly greater than the power of the adjacent frequencies, it indicates the probable existence of a rhythm having a period of that length. The statistical significance of the peak in the present

work was determined by the methods of Mercer (8) and Panofsky and Halberg (9).

Since the values of period and frequency are reciprocals, the terms period and frequency will be used interchangeably. Due to the computational methods used in the computer program, the values of the abscissa in the graphs are presented as frequency (f) in cycles per hr (cph). Values of the period length (p) in hr (h) are also given in the second scale for the abscissa. The ordinate of the spectral estimate graph represents the prevalence or power of the particular rhythm present and is expressed as the logarithm of the power (see Fig. 1). The minimal period, i.e., the highest frequency that can be detected, is determined by the interval between readings (9). In the present study, readings were taken at 1 hr intervals and the analysis covered the spectrum from 0.000 cph (infinite period) to 0.500 cph (2 hr period), in intervals of 0.005 cph. An analysis based on intervals of 0.0007 cph was later made between the frequencies of 0.025 to 0.050 cph to determine the mean and the standard deviation of the circadian rhythm. Since no significant peak appeared beyond the 0.200 cph point in any of the data tested, the graphs presented in this paper were not extended beyond that point.

Among plants showing persistent leaf movements, the Pinto bean (*Phaseolus vulgaris*) was selected as the test plant because of the ready availability of the material and the authors' familiarity with the plant (7). The plants were raised either in a long-day greenhouse or in a growth chamber with continuous light until the primary leaves were half-expanded. The plants were about 7 days old at this time. Four or 8 plants were used in each experiment. Plants, selected for their vigor and uniformity, were transferred to a growth chamber having continuous light from power groove fluorescent tubes with an illuminance of 750 ft-c at leaf level. Temperatures of 5°, 10°, 15°, 20°, and 28° were maintained within a range of ± 0.5° during the experimental period. Humidity was maintained between 50 and 65 % in the 15°, 20°, and 28° treatments, between 70 and 85 % in the 10° treatment and between 80 and 100 % in the 5° treatment. In the 5° experiments, leaf movements ceased soon after the plants were placed in the constant temperature chambers. Thus, analysis was not possible by computer techniques.

Time lapse photographic records of the leaf movements were taken for 28 days using a 24-hr clock placed in the photographic field as a time indicator. The position of the leaf image was measured with the aid of a modified Benson-Lehner Boscar. The value of 0° was assigned to the angle when the leaf was straight up, 90° when horizontal, 180° when straight down (Fig. 1). The leaf angles were measured to within 5°, providing a total of 19 points in the normal 90° to 180° excursion range of the leaf. Closer readings were not attempted because of the

increased time and the limit of accuracy. The precision in estimating the period length was limited by the 5° increments in the data, the 1 hr interval between data points and most important, by the 28 to 30 day life span of the primary leaves. Under these conditions the precision of determining the period length was in the order of 0.1 hr. Data including observation time and leaf angle were collected from either 8 or 16 primary leaves, transferred to IBM cards, and then analyzed by the computer program. Leaves designated number 1 and 2 were the primary leaves of the first plant, leaves number 3 and 4 were the primary leaves from the second plant, etc. Each experiment was repeated at least twice.

Results

Experiment 1. Four plants grown in continuous light at 28° from the time of planting were observed for 28 days. The leaf movement data of each leaf were analyzed individually. The results are presented in table I. The spectral estimates of all leaves except leaf number 2 had a significant peak in the 27 hr region. The one exception had a peak in this region, but it was not significant. Visual inspection of the plotted record showed that this particular leaf did not exhibit distinct circadian movements. However, leaf number 1, which was on the same plant and opposite to leaf number 2, did exhibit a clear circadian rhythm.

Table I. *The Significance of Various Peaks in the Spectral Estimates of the Leaf Movements of Pinto Bean Plants Grown at 28°.*

Graphs of the power spectra from which the data were obtained were plotted with the aid of the BMDX73 computer program of the U.C.L.A. Health Sciences Computing Facility. Peak locations are indicated both in frequency values and in period lengths. The significance of various peaks was tested by the methods of Mercer (8) and of Panofsky and Halberg (9). **,P = 0.05; —, not significant.

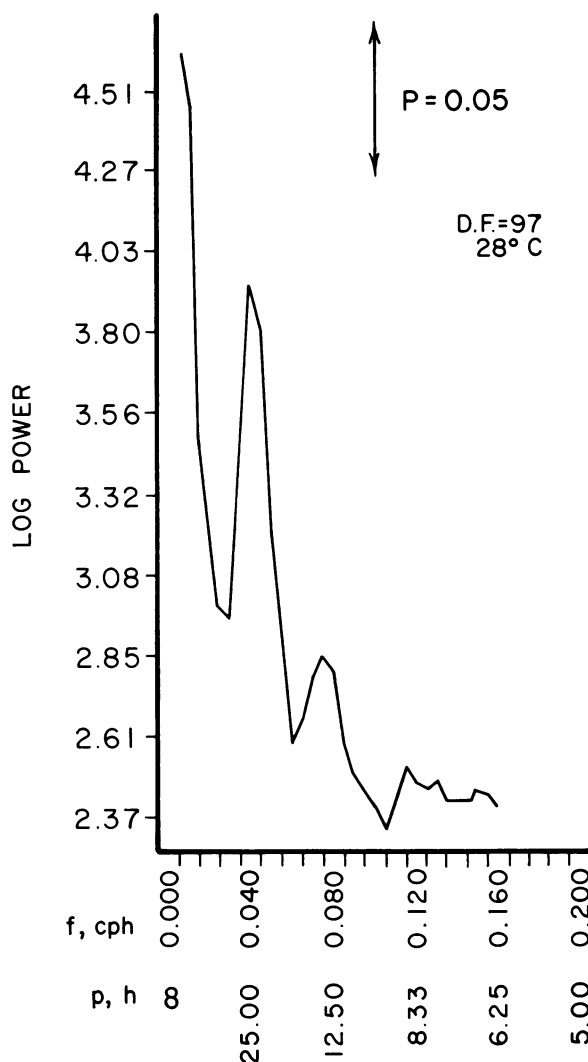| Plant No.	Leaf No.	Significance of plants located between 0.035 to 0.040 cph (27.3 hr)	0.070 to 0.075 cph (13.8 hr)	0.105 to 0.115 cph (9.1 hr)
1	1	**	—	—
	2	—	—	—
2	3	**	—	—
	4	**	—	—
3	5	**	—	—
	6	**	**	—
4	7	**	—	**
	8	**	—	—

FIG. 2. Mean of the spectral estimates of the movements of 8 Pinto bean leaves grown in continuous light at 28°. The 27.3 hr rhythm is significant beyond the P = 0.001 level and the 13.8 hr rhythm is at the P = 0.10 level (8,9). See Fig. 1 for details.

Analysis of all 8 power spectra (Fig. 2) indicated that on a group basis, the 27.3 hr period was significant beyond the P = 0.001 level, the 13.8 hr period was at the P = 0.10 level and the 9.1 hr period was not significant (8). Thus only the 27.3 hr period was highly significant. Periodicities of 2 to 4 hr in length which generally appear to be present when the records are studied visually were not indicated as significant by the analysis used. Critical examination of leaf movement data revealed that movements with periods of 2 to 4 hr were not precise and the period length varied from one period to the next. It appears from our present analytical techniques that these movements were probably random fluctuations. This however does not preclude

the possibility of the existence of a rhythm with a 2 to 4 hr period.

The frequency of the circadian component of each leaf was determined by analyzing at intervals of 0.0007 cph. The frequency values obtained for each leaf were used to determine the mean and the standard deviations of the circadian component. These values were then converted to period lengths. Since the circadian component of leaf number 2 was not significant, it was not included in the calculations. The circadian component for the 7 leaves was found to be 27.3 ± 0.2 hr.

The time of day at which the leaves reached the horizontal position was found by visual inspection of the leaf movement graphs to vary quite markedly from plant to plant and from leaf to leaf (7). In one of our plants, the phases of the 2 primary leaves were found to be within 2 hr of each other. In the other plants the opposite leaves were found to be approximately 4, 7, and 13 hr out of phase with each other. These observations were substantiated by the results of the cross-spectral analysis. Thus for Pinto beans grown under constant light and temperature from the time of planting, the 2 opposite leaves of a plant were independent with respect to phase of movement.

Experiment 2. In this experiment 8 plants were placed in a constant environment chamber set at 20°. Except for this change of temperature, the experimental conditions were identical to those of the previous experiment. In the previous experiment a highly significant peak occurred in the 27.3 hr region and a second peak occurred in the 13.8 hr region. Since Bunning and Tazawa (6) report that they were able to entrain *Phaseolus multiflorus* to short light-dark cycles at lower temperatures and since the entrainment they reported may have been due to a presence of a short period similar to the 13.8 hr period detected in our experiments, it was decided to conduct an experiment at lower temperatures such as they used and to study possible changes in the previously observed peaks. As in the first experiment, the data for all the leaves were combined and the mean curve derived from the 16 spectral analysis curves (Fig. 3). The peaks in the 27.3 and 13.8 hr region were highly significant with the 27.3 hr peak much stronger than the 13.8 hr peak. A third peak occurred at the 9.1 hr point and a fourth peak at the 8 hr point. However, these peaks were found to be just at the $P = 0.10$ level.

To test for the presence of harmonics in the spectral analyses of leaf movement data, an acceptance filter of 0.035 cph was used to decrease the peaks of the derived series to yield harmonics. The cross-spectral analysis of this derived series and that of the original data were calculated to determine if the suspect peaks were harmonics. The results obtained indicate that the peaks could not be wholly accounted for as harmonics and thus indicate the high probability of the presence of not only the 27.3 hr rhythm but also the 13.8 hr rhythm.
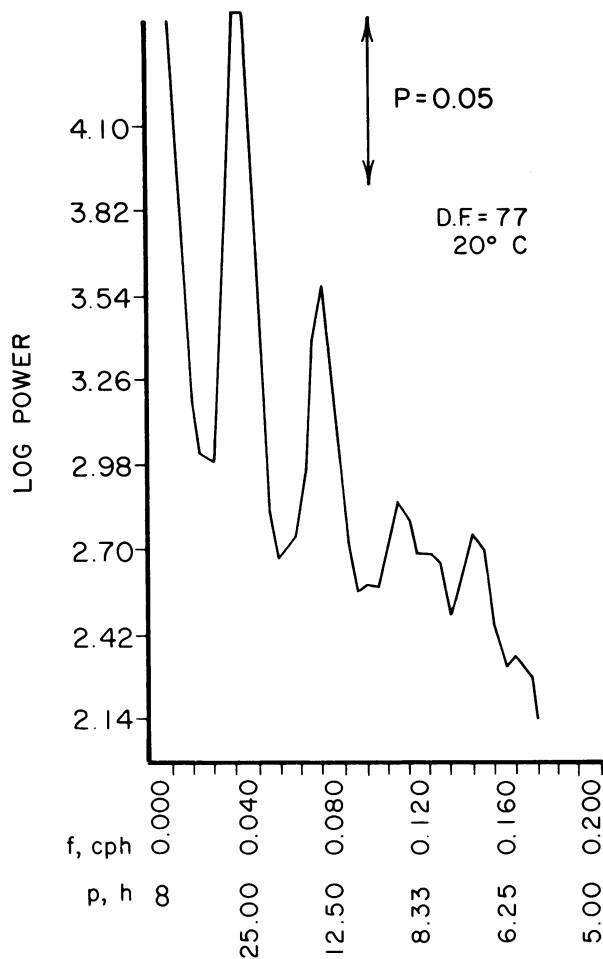

FIG. 3. Mean of the spectral estimates of the movements of 16 Pinto bean leaves grown in continuous light at 20°. For the 27.3 and 13.8 hr rhythms, $P<0.01$ (8,9). See Fig. 1 for details.

Experiment 3. The experimental conditions were identical to the previous experiments except that the temperature was set at 15°. The peak occurring at the 13.8 hr point in the 28° experiment was found to be at the $P = 0.10$ level, while in the 20° experiment, this peak was highly significant. The finding of this change and the appearance of other shorter periods in the 20° experiment led us to test the effects of a still lower temperature on the leaf movements of Pinto beans.

As was done in the previous experiments, the data from the 8 leaves were combined. The results are shown in Fig. 4. The 27.3 and 13.8 hr components were highly significant and were about equal in strength. A peak also appeared at the 9 hr point but was not significant. A fairly prominent peak occurred at the 5.9 hr point and was just within the limits of significance. It can be noted, however, that the "noise" level depicted in this graph had risen in the region of shorter periods and the sig-
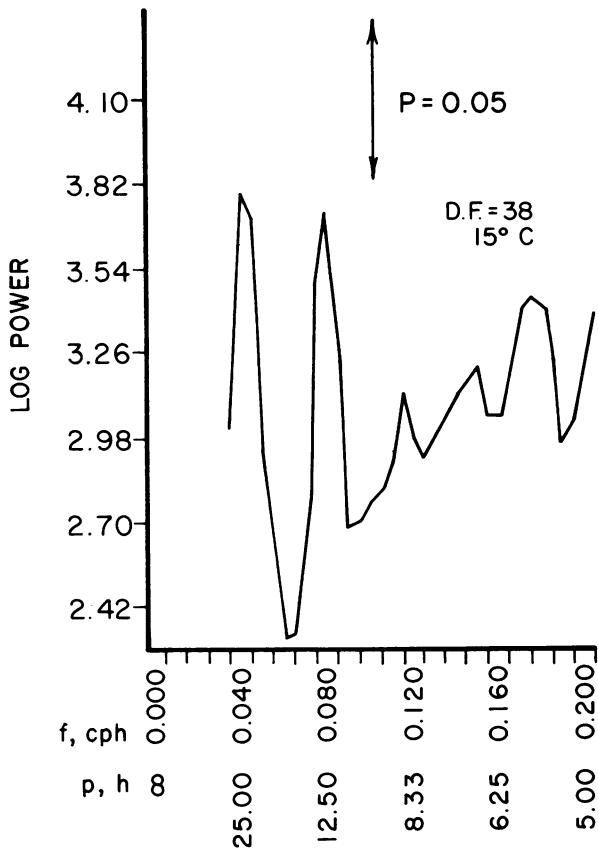
FIG. 4. Mean of the spectral estimates of the movements of 8 Pinto bean leaves grown in continuous light at 15°. The 27.3 and 13.8 hr rhythms are significant beyond the P = 0.01 level. The 5.9 hr component is at the P = 0.10 level (8, 9). See Fig. 1 for details.

nificance of the shorter period peaks may have been obscured by this rise.

Experiment 4. To test further the effect of temperature on the various components of the leaf rhythm, 8 Pinto bean plants were placed in a 10° environment. Other experimental conditions were the same as for the previous experiment. Circadian components were not found using power spectral analysis. Bunning and Tazawa (6) found a similar failure of the circadian component at 10° for *Phaseolus multiflorus*. However they did report a predominance of high frequency movements. In our Pinto beans, the power spectra did not indicate the presence of a significant rhythm nor a predominance of high frequency movements. Bunning and Tazawa's method of analysis was quite different from ours and could account for the differences in the interpretation of the results.

Discussion

The results obtained by computer analysis indicate that the primary leaves of Pinto beans have a precise circadian rhythm. In the presence of a "Zeitgeber" such as a light-dark cycle, it is well known that movements of leaves are synchronized with each other. However, the rhythms of leaves on plants grown in constant conditions are not necessarily synchronized and can be independent of each other in terms of the phase angle. Not only can the leaves of different plants move independently but the primary leaves opposite each other on the same plant can be out of phase with each other. These results support the thesis that the timing mechanism involved in rhythmicity may reside in individual cells (3). In our Pinto beans the cells of one pulvinus can be in phase with each other yet be independently phased from the cells of another pulvinus.

One may conclude from the evidence presented that there are in Pinto beans free running rhythms of different lengths and that these only manifest themselves at different temperatures. At 28°, only a 27.3 hr component was evident to any great extent, whereas at 20°, a 13.8 hr component also became highly significant. At 15°, the 13.8 hr component was as strong as the 27.3 hr component and a third peak, a 9 hr component, also increased though not to the high significance of the former 2. Since the 13.8 hr component is also the second harmonic of the 27.3 hr circadian component, the peak representing the 13.8 hr rhythm may be due in part to leaf movements that deviate from the sinusoidal circadian movements. A sharp dip in the movement or a large change in the period length are 2 examples of deviations that contribute to the second harmonic peak. If these deviations are large and frequent, a significant peak can arise at the second harmonic in the power spectrum. Peaks can also occur at other harmonics for the same reason. However, as stated previously, a test for harmonics indicated that such peaks were not strictly due to harmonics.

As the temperature was lowered, the high frequency components (9 and 13.8 hr periods) became more prevalent. Bunning noted short period movements of *Phaseolus multiflorus* leaves when the plants were subjected to 12° (5). He attributed these short-period movements to a premature relaxation of the tension phase in the rhythm. These movements of *P. multiflorus* were probably similar to those of our Pinto beans grown at 15°. Whether these short-period movements in Pinto beans were due to a relaxation type of oscillator or to a pendulum type could not be determined from analysis made on our data. However these short period rhythms were free running since at no time during these experiments were the plants subjected to an alternating light-dark cycle.

A comparison of the power spectra of leaf movements of Pinto bean plants grown at different temperatures showed an increase of the "noise" level in the very short period region (9 hr or less) at the lower temperatures. Under continuous light and low temperature, the Pinto bean leaves had very

short period movements that were not strictly periodic. It is very possible that when Bunning and Tazawa (6) were testing the effect of temperature on leaf movements, they were able to entrain the leaves of *Phaseolus multiflorus* to the very short light-dark cycles because of the presence of high frequency random movements at low temperatures.

Acknowledgments

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