Plant Physiol. (1970) 46, 99–102

Circadian Rhythm of Leaves of *Phaseolus angularis* Plants Grown in a Controlled Carbon Dioxide and Humidity Environment[1]

Received for publication October 1, 1969

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ABSTRACT

Leaf movements of primary leaves of *Phaseolus angularis* Wight. were studied in an environment with controlled levels of CO_2, relative humidity, temperature, light, nutrient concentrations, and water tension. Rhythmic circadian movements and irregular short period movements were evident as the leaves unfolded and persisted during development of the leaves. The mean period in rhythmic circadian movement was 27.3 hours with no significant differences in period between plants of the same or different experiments. The leaf movements of separate plants were not closely synchronized.

A system is described for growing plants for extended periods while collecting data with time lapse photography. The system was developed to minimize disturbances to the plants.

The occurrence of leaf movements in plants grown from the time of seeding in an environment free of any recognized fluctuation which could stimulate leaf movements supports the hypothesis that leaf movement rhythms originate spontaneously within the plant.

Circadian rhythms have been observed in many types of organisms and are especially conspicuous in the movements of the primary leaves of many plants. The movement rhythms are present under constant light and temperature conditions (2, 4, 7). Under continuous light these rhythmic movements have been recorded for a period of 4 weeks (8). It is assumed that these circadian movements are endogenous to the plant and that the "internal oscillator" (2) is expressed without any environmental stimuli. However, Bünning (2) observed that circadian leaf movements are frequently not evident at the time of seedling emergence and suggested that the young plants require some fluctuation in light or temperature to initiate the movements. It has been recognized that movement rhythms of *Phaseolus*, once initiated, are sensitive to temperature or light alterations which can significantly phase shift or reset the rhythm without changing the period of subsequent movements (5, 9, 10).

The plotted records of circadian movements of the leaf are not smooth sinusoidal curves but tend to be superimposed with irregularities, which represent upward and downward movements of small amplitude with periods of less than 1 hr (11). These irregularities, which are observed consistently in all movement records, may result from environmental fluctuations that trigger these responses. Bünning *et al.* (3) reported that these short period movements can become rhythmic when an intense environmental fluctuation is imposed upon the plants.

Environment has a profound effect on leaf movements, but its role in initiating and maintaining these movements is uncertain. Although previous investigations have been conducted under uniform light and temperature conditions, the control of other environmental factors has not been emphasized. Perhaps fluctuations of environmental factors such as CO_2, relative humidity, nutrition, or water availability initiate and maintain leaf movements. The procedures for growing plants described herein and the facilities at the Biotron at the University of Wisconsin provided an opportunity to study leaf movement responses in an environment with a minimum amount of fluctuation in relative humidity, CO_2, water tension, and mineral nutrition within a carefully controlled temperature and light room.

MATERIALS AND METHODS

The adzuki bean, *Phaseolus angularis* Wight., was selected in preference to the kidney bean, *Phaseolus vulgaris* L., because it is smaller in size and the unifoliate leaves are flatter, which makes it more desirable for time lapse photography of the leaf movements. Growth of this bean species was studied to determine the levels of temperature, light, humidity, and nutrition that provide optimal development of the young plants.

A system was developed to minimize fluctuations in environmental factors. The plants were grown from seed in cubical blocks (52 cm^3) molded from acrylonitrile modified cellulose wood fiber, BR-8 (American Can Co.) and then saturated with quarter-strength Hoagland's nutrient solution (6). The exposed surfaces of the blocks were covered with black plastic to reduce water loss. By means of a siphon system, a constant liquid level was maintained at the base of the blocks by additions of distilled water to replace water lost by transpiration and evaporation. This provided a nonfluctuating moisture and nutrition level. The plants were grown in a 75- × 40- × 45-cm airtight Plexiglass chamber placed within a controlled environment room of the Biotron. The Plexiglass chamber protected the plants against CO_2 and humidity fluctuations while individuals were in the room. Rubber glove inserts permitted adjustment of the plant orientation following emergence. Continuous light at 600 ft-c intensity was provided by warm white fluorescent lamps supplemented with tungsten lamps. The light was monitored continuously with a selenium light sensor which was calibrated with a Kahl light meter. A spectral distribution of radiant energy obtained with a spectroradiometer is shown in Figure 1. A temperature of 24 C within the chamber was maintained through tem-

[1] Part of Ph.D. thesis of D. K. Alford. This research was supported in part by a National Aeronautics and Space Administration Institutional grant. Published with permission of the Director, Research Program Division, College of Agricultural and Life Sciences, Madison, Wisconsin.

perature control of ambient air of the room. A resistance-type probe was used for continuous temperature monitoring. Changes in CO_2 and relative humidity were minimized by continuous addition of 300 cm³/min of dry compressed air from cylinders containing CO_2 concentrations near 0.040%. This added air was humidified by passage through water. A saturated sodium chloride salt bath was placed in the chamber to control the relative humidity at approximately 85%. The CO_2 concentration was monitored continuously with an infrared CO_2 analyzer calibrated at least every 48 hr with standard CO_2 gas. A continuous measurement of relative humidity was provided by a $LiCl_2$-type probe calibrated prior to each experiment with a ventilated psychrometer. Recorders provided continuous records of illumination, temperature, relative humidity, and CO_2 in the chamber. A diagram and photograph of this system are shown in Figures 2 and 3, respectively.

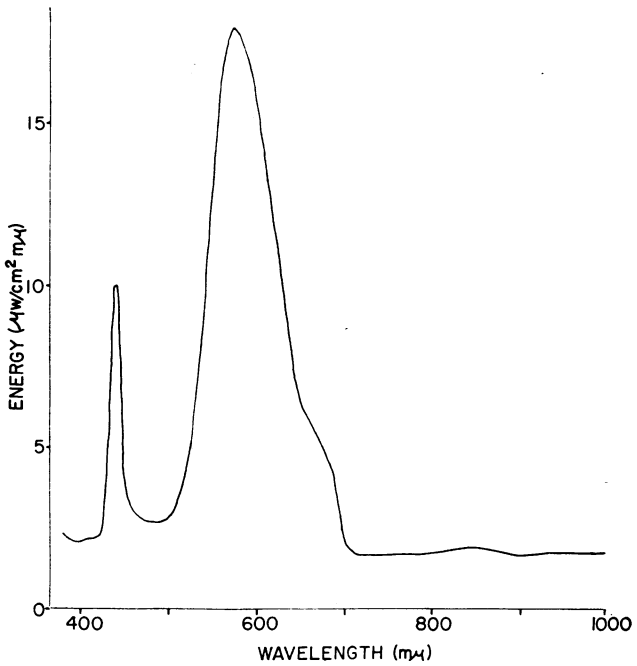

FIG. 1. Spectral distribution of light energy at the leaf surface of plants at 600 ft-c.

Records of leaf movement responses were obtained from four plants in two experiments and from three plants in a third experiment. Two seeds were planted in each block and as the leaves of the emerging seedling unfolded, the blocks were thinned to one plant each and the cameras were set into operation. The growing plants were disturbed only for thinning just following emergence and for removal of the apical meristems and young trifoliate leaves 8 days after leaf unfolding. Entrance into the room was avoided if possible because the light intensity decreased 10 ft-c at the plant level when one entered the room.

A 16-mm camera provided time lapse photographs of the leaf positions at 6-min intervals. The camera was located outside the chamber with the lens fitted into an airtight rubber-lined opening in one wall.

The leaf movement response was determined by measuring, with the aid of a protractor, the leaf angle of each plant on projected images of the film. The measured angle was defined by two lines radiating from the secondary pulvinus at the junction of the petiole and leaf blade. One line extended parallel to the stem axis, and the other line passed through the leaf tip (Fig. 4). The tabulated angular readings were then plotted against time at 6-min intervals.

The period of the rhythm was determined from the plottings of the movement data by calculating the time interval between successive points marked on the downward phase of the movement cycle, midway between maximal upward and downward positions of the blade. Most investigators have used the time of maximum downward leaf position as the point of reference. However, that method was found to be less precise than the method described herein.

RESULTS AND DISCUSSION

The results indicate that both rhythmic circadian movements and short period movements of primary leaves of *Phaseolus*

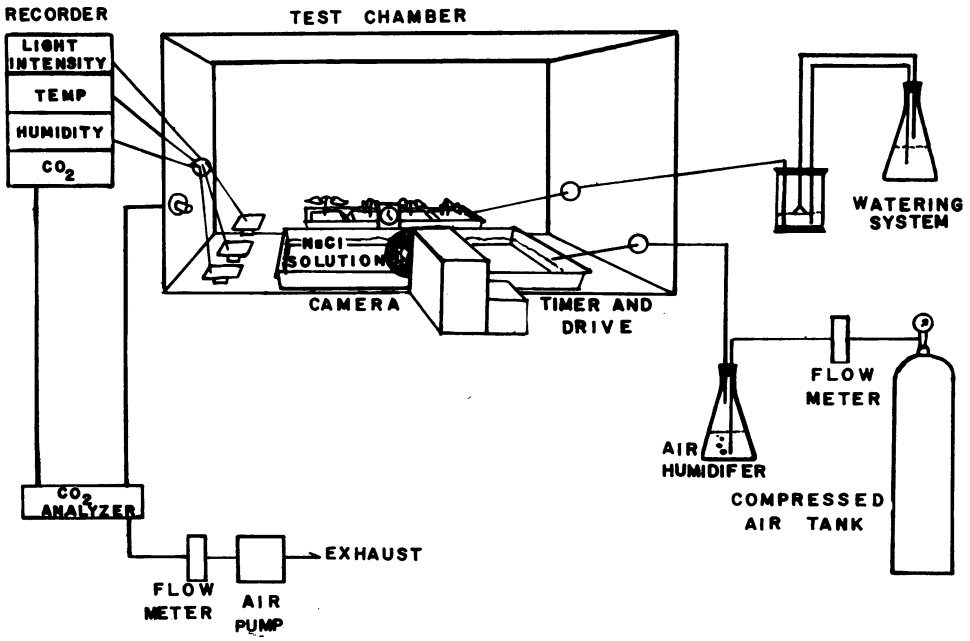

FIG. 2. Uniform environment system for leaf movement studies. The test chamber is maintained in a constant temperature and illumination room of the Biotron.

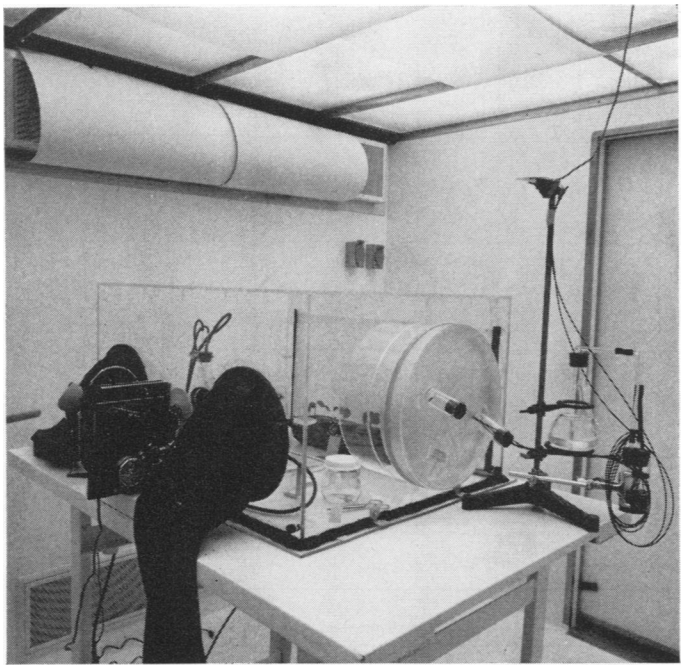

FIG. 3. Plexiglass test chamber in the Biotron room.

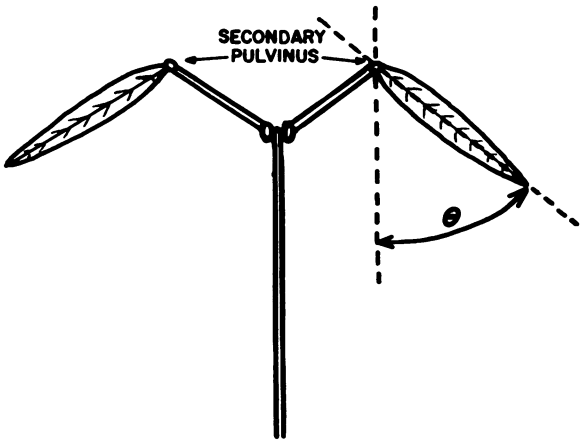

FIG. 4. Leaf angle (θ) measured on the projected image to record upward and downward movements of *Phaseolus* leaves.

originate spontaneously as the leaves of the emerging seedling unfold and continue free running when the plants are grown under a controlled environment.

The small sudden fluctuations and gradual changes in environmental factors in these experiments did not phase shift or synchronize subsequent leaf movements. Fluctuations in environmental parameters together with leaf movement responses of four leaves are shown in Figure 5.

During this experiment there were gradual and consistent changes in both CO_2 and relative humidity. The relative humidity increased from 80 to 87% because of water loss from the enlarging plants. The CO_2 level decreased as a result of CO_2 fixation by the plants. During the first 9 days the CO_2 concentration decreased from a level of 0.040% to 0.025%. After removal of the apical meristem and trifoliate leaves, CO_2 fixation was reduced, and thus the CO_2 level in the chamber increased slightly during the final 5 days. It should be noted that an environmental control system could have been designed to provide frequent adjustment resulting in greater uniformity in relative humidity and CO_2 concentrations. However, the cycling of a control system of this nature was considered undesirable since it could have entrained leaf movement rhythms. The control system utilized in this study was considered preferable since it avoided these rhythmic adjustments.

The maximal variation in temperature was ±0.5 C and in light intensity ±15 ft-c during the 14-day period (Fig. 5). Although the light intensity was carefully controlled, momentary fluctuations in light intensity were observed when equipment was placed into operation within the Biotron. The magnitude of these changes could not be accurately detected by the selenium light sensor; therefore, they were not included with the plotted light intensity data. Leaf movement responses were not synchronized to or phase shifted by these momentary light fluctuations.

When the apical mersitem and trifoliate leaves were removed (arrow, Fig. 5), there was a sudden 27% increase in CO_2 levels and a 5% decrease in relative humidity caused by an influx of room air into the Plexiglass chamber when the rubber gloves were withdrawn. Although there appears to be some decrease in amplitude of leaf movements subsequent to these fluctuations, it is important that fluctuations in these parameters did not phase shift or synchronize subsequent movement cycles. The removal of the apical meristem and trifoliate leaves or simultaneous environmental changes did cause an abrupt downward movement of the petiole and blade through activity of the primary pulvinus at the junction of the stem and petiole. This was generally a temporary response as the petiole returned to its approximate original position within 24 hr.

Circadian and other short period movements have been observed for 23 days from the time of leaf unfolding. The amplitude of the movements decreased considerably with time. This decrease was not related to leaf senescence since an increase in movement amplitude was stimulated again with two successive dark periods after more than 3 weeks of growth.

The amplitude and regularity of the short period upward and downward movements changed with the leaf position and age of the plant (Fig. 5). When the leaf blade was in an upward position, the movements were of small amplitude. However, when the blade was down, or in the process of moving upward, the short period movements were of larger amplitude. At certain times the short period movements were rhythmic. This was observed during the first 7 days after leaf unfolding and only while the leaf blade was in an upward phase of the movement cycle. Spectral analysis by computer indicated that there was a dominant movement component with a period of approximately 60 min during this early growth. These rhythmic upward and downward movements may be controlled by the same mechanisms that control the circumnutation movements of the plants for during the first 2 or 3 days after leaf unfolding, a circumnutation movement of the leaf tip was often evident and accounted for many of the measured upward and downward movements with a period of about 1 hr. The circumnutation movement was not evident after this initial period.

In this study a precise estimation of the period of the circadian movement of *Phaseolus* leaves was possible. The period of successive cycles within plants differed by as much as 4.5 hr, which was considerably greater than the errors in measurement, but the mean cycle length for an individual plant varied only ±0.7 hr from the 27.3 hr mean of all plants. An analysis of variance with an *F* test indicated that the variation between plants was not significant at the 0.05 probability level. For the three experiments, the variation in the mean period was only ±0.1 hr (Table I). It was observed also that there was no significant change in length of cycles as the leaves aged (Table II).

Although the circadian leaf movements for the two leaves on the same plant were usually closely in phase, they could be several hours out of phase, as observed previously by Hoshizaki

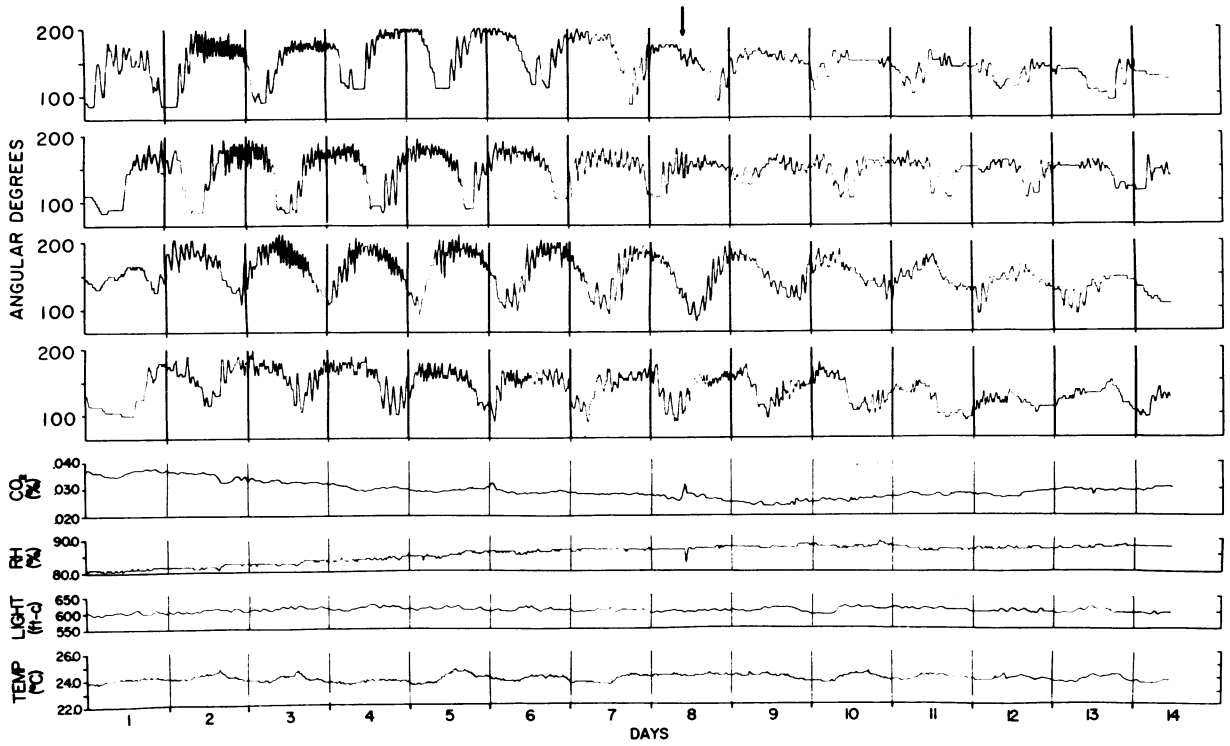

FIG. 5. Movements of individual primary leaves from four *Phaseolus* plants recorded from the time of leaf unfolding, together with environmental data recorded simultaneously. The arrow indicates the time of removal of apical meristems and trifoliate leaves.

Table I. *Mean Period and Standard Deviation of Movement Cycles for Phaseolus Plants in Three Experiments*

Experiment	No. of Plants	Mean Period	Standard Deviation
		hr	*hr*
A	4	27.4	1.2
B	4	27.4	1.1
C	3	27.2	0.8

F value for experiments, 0.24; not significant.

Table II. *Period of Successive Movement Cycles for Phaseolus Plants*

Plant	Successive Cycles										
	1	2	3	4	5	6	7	8	9	10	Average
	hr										
A	27.9	27.4	27.9	26.9	27.9	28.1	28.1	28.8	27.3	26.8	27.7
B	28.4	27.4	27.3	28.5	28.0	25.6	30.3	26.1	27.7	25.4	27.5
C	23.5	25.2	26.6	26.8	26.5	27.8	28.2	27.3	26.4	28.5	26.7
D	28.6	28.3	28.1	26.9	28.2	27.7	28.5	30.2	26.8	26.8	28.0
Average	27.1	27.2	27.5	27.3	27.7	27.3	28.8	28.1	27.1	26.9	

F value for plants, 1.92; not significant. *F* value for successive cycles, 0.77; not significant. Standard deviation, 1.2 hr.

and Hamner (8). Likewise, the movement of one plant was not necessarily synchronized with the movement of another plant (Fig. 5). It was suspected that the circadian clock of each plant may be set separately as the young seedlings emerge from the edaphic to the ephatic environment with the accompanying changes in temperature and light. However, temporal relationships between the time of emergence and the first downward leaf movement were not found.

Although there were no apparent correlations of movement response to the environmental factors controlled in these experiments, the possibility that fluctuations in certain natural geophysical phenomena such as cosmic radiation, magnetic fields, and barometric pressure may have stimulated these movements cannot be completely discounted. It has been reported that circadian rhythms are synchronized with fluctuations in geophysical factors (1). Data for fluctuations in these factors were collected during the course of this study, and correlation analyses are currently being conducted.

Acknowledgments—We express our gratitude to the staff of the Biotron for their valuable assistance in this study.

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