Chronobiology of Aging in *Albizzia julibrissin*1

I. AN AUTOMATED, COMPUTERIZED SYSTEM FOR MONITORING LEAFLET MOVEMENT; THE RHYTHM IN CONSTANT DARKNESS

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ABSTRACT

We are using leaflet movements in *Albizzia julibrissin* as a model system for investigating the chronobiology of aging. To monitor leaflet movements during long dark periods with adequate temporal and spatial resolution, we designed an automated, computerized phototactic monitoring system. Each of 12 leaflet pairs was positioned in an individual light-proof container, with one leaflet immobilized. The angle of the mobile leaflet was monitored by a photosensor array using a low intensity infrared beam. Leaflet position was determined by custom-developed software, using information on the shading patterns of the sensors. Data on leaflet angle as a function of time were collected and stored on a floppy disc and then printed in numerical and graphical form.

Oscillations of young, middle-aged, and old leaflets persist during 7 days of darkness with a periodicity close to 24 h. Period length appears to be age-independent, but rhythmic wave form is age-dependent. The older the leaflet, the earlier and more completely it opens and the less completely it closes.

Our knowledge of biological timekeeping has increased dramatically during the past few decades. It is now evident that the biochemistry, physiology, and behavior of virtually all eukaryotes change during 24-h environmental cycles (3, 11). Many of these oscillations persist under free-running (constant) conditions, since they are controlled by a circadian biological clock.

Circadian rhythms characteristically alter as an organism ages (15). Such alterations have been described in humans (20), other mammals (10, 14), higher plants (13), and fungi (12). Amplitude of the oscillation often decreases during the aging process; in some cases, rhythmicity disappears entirely (15). Phase relationships between different rhythms in a single individual may also change with age (10). In addition, rhythmic frequency changes with age in some rodent species (14) although it remains constant in others (8).

We are using leaflet movements in the leguminous plant *Albizzia julibrissin* as a model system for investigating the chronobiology of aging. *Albizzia* bears doubly compound leaves that are divided into paired pinnae, in turn subdivided into paired pinnules (leaflet) (see Fig. 1a in Ref. 16). Paired pinnae and pinnules usually separate from one another (open) in the light and fold together (close) in the dark, but leaflet angle oscillates with a circadian periodicity under free-running conditions (reviewed in 16).

*Albizzia* has certain unique advantages for studying the chronobiology of aging. Each plant continually produces new leaves at the apex of the continually elongating main axis. Nevertheless, each individual leaf has a limited life span. When it reaches a certain age and developmental state, it senesces and dies. Thus, a single plant provides a large amount of clonal material of different ages. Problems of genetic variability inherent in experiments with a population of organisms are thereby avoided.

We already demonstrated that leaflets of different ages move at different rates when transferred from light to darkness (9). To investigate rhythmic movements during longer periods of time, we built an automated, computerized system capable of monitoring leaflet angle with high temporal and spatial resolution. We now describe our monitoring system, and present data on rhythmic patterns of leaflets of different ages.

MATERIALS AND METHODS

**Plant Materials.** *Albizzia julibrissin* Durazz, plants were grown from seed with 16-h light:8-h dark cycles (cool white fluorescent, 55 μmol m⁻² s⁻¹) at 24°C. The plants were 12 to 18 months old and had about 25 mature leaves when leaflets were excised for experimental use. Leaf age is designated as in Lee and Satter (9): the three uppermost fully expanded leaves are called young, the next seven are called middle-aged, and the next seven are called old. We did not use immature leaves at the apex that remain closed continuously or very old leaves at the base that were poorly pigmented.

**Experimental Conditions.** Our monitoring system was operated in a temperature-controlled, physiologically dark room (green safelight illumination). A red light source was available in the same room.

Young, middle-aged, and old leaflet pairs were excised from a 1- to 2-year-old plant at the end of the photoperiod, floated on water in Petri dishes, and then exposed to red light (20 μmol m⁻² s⁻¹) for 5 min. The red light source contained two banks of two fluorescent tubes (General Electric F 20T 112 WW) wrapped in three layers of red cellophane. One bank was positioned above the leaflets and one below, so that both pulvini in a closed leaflet pair would be irradiated similarly. Red irradiation establishes a high level of Pfr in the tissue, as required for persistence of rhythmicity during DD2 (17). The leaflet pairs were maintained in physiological darkness immediately following the red irradiation. Each leaflet pair was mounted in an individual monitoring box (Fig. 1) filled with 50 mm sucrose, also required for persistence of rhythmicity during DD (17). The sucrose solution was changed daily to minimize bacterial contamination.

**RESULTS AND DISCUSSION**

**Methods for Monitoring Leaflet Movement.** Several methods have been used in the past to monitor leaf movements in nyctinastic (night closure) plants. These include: kymograph and lever systems, strain gauge methods, time-lapse photography, and

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2 Abbreviation: DD, uninterrupted darkness.
EQUIPMENT FOR MONITORING LEAF MOVEMENT

and photoelectric systems (reviewed in 16). We decided to use a photoelectric system rather than a kymograph and lever or strain gauge to minimize mechanical interference with the movements. This is particularly important for Albizzia leaflets, since their motor organs (pulvini) are small (diameter about 0.5 mm, length about 0.4 mm). A photoelectric system is preferable to time lapse photography since the data can be digitized more readily for storage and analysis. We designed a new photoelectric system rather than use our former system (1) to obtain improved angular resolution. Our new system incorporates 18 phototransistors in each monitoring unit whereas our earlier system used only one phototransistor. Superior angular resolution is essential for investigations of age-dependent changes in wave form and frequency, since such changes may be small and subtle.

Description of Our Equipment. We designed a monitoring system that measures the angle between excised paired leaflets, and stores the data in a form suitable for computer analysis. We have built 12 units, each containing its own monitor and sharing a system controller and microcomputer with other units, as described below. Additional units can be added as needed. We offer to consult with anyone who wants to duplicate our equipment.

Monitor. One leaflet of each pair is immobilized on top of a wet sponge, while the other leaflet is allowed to move freely (Fig. 1). As the angle changes from 0 degrees (completely closed) to 180 degrees (completely open), the tip of the mobile leaflet moves through a semicircle. A 3-mm diameter IR emitting gallium arsenide diode source (TIL 38, Texas Instruments) is installed on one side of the leaflet pair, while an array of 18 small phototransistors (TIL 604, Texas Instruments) are arranged on the opposite side in a semicircle and function as IR detectors. The set-up is housed in a light proof container.

In our first model, the change in angle of the moving leaflet was detected by the successively timed shading of adjacent transistors. The signal generated by each transistor was amplified, with gain set so that the output was higher than a specific reference voltage when the transistor was not shaded. When the transistor was shaded, however, the output dropped well below the reference voltage. The output of each amplifier was constantly compared to the reference voltage via a comparator. The data were then digitized, i.e. '0' was generated when the transistor was not shaded and '1' was generated when it was shaded. All of the data presented in this paper were obtained with this system.

Two unexpected problems arose. (a) Reflection of the IR beam by the sucrose solution on which leaflets floated increased the voltage across the transistors closest to the solution. Consequently, the system had to be recalibrated during the course of an experiment. (b) Occasionally, the shadow of the leaflet was positioned between two phototransistors such that neither was shaded sufficiently for its output to drop below the reference voltage. To prevent these problems, we redesigned our monitoring units based on a new concept that relies heavily upon software.

In our new design, we use an analog-to-digital converter to directly digitize the output of each phototransistor. The computer records all the readings. The information is stored but is not processed until an experiment has been completed. At this time, a reference voltage is calculated for each sensor by averaging the 10 highest output voltage readings of the sensor during the experiment. The 'degree of shading' of each phototransistor is then calculated for each measurement time by comparing the signal output with its reference value. Leaflet position is determined by using information on the shading patterns of all sensors. Since the shadow created by the leaflet may be broader than a single phototransistor, the voltage across more than one transistor may be affected at the same time. By carefully comparing the degree of shading of neighboring transistors, any subtle movement of the leaflet can be detected. The whole process is done automatically with custom-developed software.

A major advantage of our new modification is that angular resolution is not limited by the number of transistors in each monitor. Leaflet angle is monitored with a resolution of 2° to 3°.

System controller. The system controller contains a dedicated 8-bit microcontroller operating under custom-developed firmware. The controller processes incoming data from the monitoring circuit and records information about the operating environment. A user-interface control panel, a real-time clock, and a digital temperature sensor are interfaced to the system controller. The modular design enables the system to be easily adapted to different computers.

Microcomputer. Data are read by an Apple IIe computer and stored on a floppy disc, together with the actual time of each measurement and the temperature at these times. All relative interfaces between different section circuits are controlled by this computer via supplementary hardware. We use the same computer for software development and data analysis.

Printer. The data are printed in numerical and graphical form on an Okidata Microline 92 Printer.

Leaflet Movement during a Prolonged Dark Period. Computer-generated graphs of the movements of young, middle-aged, and old leaflet pairs during 160 h DD, temperature = 25°C, are shown in Figure 2. Leaflets of all three age groups oscillate with circadian periods. Since the free-running period length is close to 24 h, it was important to consider whether a perturbation synchronized to solar time might have entrained the oscillations. We customarily remove the sucrose solution on which the leaflets float and replace it with fresh solution at about 24-h intervals; thus, we questioned whether this manipulation acted as a Zeitgeber for rhythmic entrainment. To test this possibility, we changed the solution at random intervals; period length was unaffected. The solution change was the only perturbation that was normally synchronized to solar time.

The movement patterns of young, middle-aged, and old leaflets during the first rhythmic cycle are shown on a more highly magnified time scale in Figure 3, while data from several experiments are summarized in Table I. Note that the amplitude of the oscillation is larger for old leaflets than for middle-aged and young leaflets. Rhythmic amplitude in other organisms generally decreases with age, although certain exceptions have been noted (10).

Leaflet age has a large effect on rhythmic wave form. The older the leaflet, the earlier and more completely it opens, the less completely it closes, and the longer the interval between the beginning of opening and the end of closure. Thus, old leaflets spend a larger part of the rhythmic cycle in the open configura-

FIG. 1. Schematic drawing of one of our units for monitoring the movement of Albizzia leaflets. See text for a description of the equipment.
tion from light to darkness. Inability to close might be due in part to structural modifications (4). The xylem becomes more heavily lignified and cellulosic vascular walls thicken as *Albizia* leaflets age; these changes would impose mechanical constraints upon the movements. Mitochondrial shape and plastid ultrastructure also change as leaflets age; these changes might signify alterations in metabolism. In addition, membrane-localized ion pumps and/or leaks might change as leaflets age. Leaflet movements are dependent upon differential changes in the turgor of cells in the motor organ. Motor cell turgor changes, in turn, are dependent upon fluxes of $K^+$, $Cl^-$, $H^+$, and other ions, which regulate water movement by osmosis (7, 16, 18, 19). Decrease in the rate of $H^+$ secretion (5) and increase in membrane leakiness (6) and in membrane microviscosity (2) have been reported in aging leaves of other plants.

Age-dependent changes in rhythmic wave form have been described in other organisms (10, 13) as well as in *Albizia*. However, age-dependent changes in driven rhythms do not indicate whether the time-keeping mechanism changes with age. If the clock changes with age, some of its measurable properties might change. Rhythmic period length is a property of the clock that does not appear to change with age in *Albizia* (Fig. 2). Rhythmic phase shifting is another property of the clock. We are currently investigating whether it is affected by age; results will be presented in a later paper in this series.

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**LITERATURE CITED**


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**Table 1. Effects of Age on Several Parameters of the Leaflet Movement Rhythm in *Albizia***

Data are from the first 30 h of DD (0 h = the beginning of DD). Data are represented as mean ± SD, n = 12. Statistically significant differences (probability >95% by Student $t$ test) for a given parameter are indicated by different letters (a, b, c).

<table>
<thead>
<tr>
<th>Age</th>
<th>Opening to Maximum</th>
<th>Maximum Angle</th>
<th>Amplitude (Max. Angle–Min. Angle)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beginning</td>
<td>Minimum</td>
<td></td>
</tr>
<tr>
<td>Young</td>
<td>6:55 ± 13a</td>
<td>6h:45 ± 16a</td>
<td>120 ± 8a 23 ± 4a 98 ± 8a</td>
</tr>
<tr>
<td>Middle</td>
<td>5:47 ± 14b</td>
<td>17:55 ± 21b</td>
<td>139 ± 4b 29 ± 2a 111 ± 4a</td>
</tr>
<tr>
<td>Old</td>
<td>4:42 ± 12c</td>
<td>19:55 ± 28c</td>
<td>172 ± 4c 45 ± 5b 127 ± 6b</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Computer-generated graphs of rhythmic movements of young, middle-aged, and old leaflet pairs during 6.8 d in darkness; $T = 25°C$. (Note that old leaflets open to 180°, the maximum possible angle of leaflets that are floating on solution, and remain at this angle for several hours during the first four cycles.)

**Fig. 3.** Computer-generated graphs of rhythmic movements of young (Y), middle-aged (M), and old (O) leaflets during 30 h in darkness; $T = 25°C$. 

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**Acacia lophantha** also close different letters by oscillating (probability $Y$, middle-aged $M$, young $Y$).