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Circadian Rhythms in Photosynthesis[1]

Oscillations in Carbon Assimilation and Stomatal Conductance under Constant Conditions

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ABSTRACT

Net carbon assimilation and stomatal conductance to water vapor oscillated repeatedly in red kidney bean, *Phaseolus vulgaris* L., plants transferred from a natural photoperiod to constant light. In a gas exchange system with automatic regulation of selected environmental and physiological variables, assimilation and conductance oscillated with a free-running period of approximately 24.5 hours. The rhythms in carbon assimilation and stomatal conductance were closely coupled and persisted for more than a week under constant conditions. A rhythm in assimilation occurred when either ambient or intercellular CO_2 partial pressure was held constant, demonstrating that the rhythm in assimilation was not entirely the result of stomatal effects on CO_2 diffusion. Rhythms in assimilation and conductance were not expressed in plants grown under constant light at a constant temperature, demonstrating that the rhythms did not occur spontaneously but were induced by an external stimulus. In plants grown under constant light with a temperature cycle, a rhythm was entrained in stomatal conductance but not in carbon assimilation, indicating that the oscillators driving the rhythms differed in their sensitivity to environmental stimuli.

The availability of light changes more rapidly and predictably over the course of a day than the level of any other resource essential for photosynthesis. Because the daily rhythm of light and darkness is so predictable, it is frequently used as the signal to entrain endogenous circadian rhythms. Circadian rhythms are ubiquitous among eukaryotic organisms and regulate a wide range of physiological and behavioral processes (5, 11, 25). Circadian rhythms have been described extensively in plants, but few studies have considered the role these rhythms may play in modifying photosynthetic rates in vascular plants. Circadian rhythms could play a potentially important role in coordinating photosynthetic activity with diurnal changes in light availability.

Evidence from several approaches indicates that circadian rhythms influence photosynthetic processes. Rhythms in stomatal conductance are well documented and were described by Francis Darwin almost 100 years ago (9). More recently, rhythms in leaf conductance and stomatal opening have been recorded under constant light in intact leaves (16, 18, 20) and in isolated epidermal peels (14). Circadian rhythms in stomatal conductance could directly affect rates of carbon assimilation by limiting the flow of CO_2 into leaves.

In addition to their influence on stomatal conductance, circadian rhythms may regulate photosynthesis at other levels. Algae lack stomata, yet circadian rhythms in photosynthesis have been reported in several species of algae (3, 15, 27, 29). Among algae, processes under circadian control that may influence photosynthesis include photosystem II activity (22) and chloroplast movement (3). Among higher plants, light-induced electron flow (19) and carbohydrate partitioning (4, 6) may be influenced by circadian rhythms. Gas exchange studies with several species have indicated diurnal changes in CO_2 compensation points and dark respiration rates (7, 17, 21). Circadian rhythms in net assimilation under constant light with constant ambient CO_2 levels have been reported for several species, including barley (10), peanut (21), and *Chenopodium* (7).

These reports indicate that the intrinsic photosynthetic activity of vascular plants, aside from stomatal effects, may be under circadian regulation. Many aspects of this phenomenon, however, are still unclear. Perhaps most importantly, no one has reported a circadian rhythm in carbon assimilation in higher plants under conditions of constant intercellular CO_2 levels, leaving the relative influence of stomatal and nonstomatal processes on rhythms of assimilation in question. Other unknown aspects of the relationship between rhythms in stomatal conductance and carbon assimilation include the phase relationship of the rhythms and the growth conditions necessary for their expression.

MATERIALS AND METHODS

We studied photosynthesis with a computer-controlled gas exchange system modified to maintain constant environmental and/or physiological conditions over several days. The gas exchange system used in these experiments evolved from the system of Field *et al.* (12). Calculations relating to photosynthesis (1, 13, 28) and a description of the materials used in this system (12, 13) are available elsewhere. Four components of the gas exchange system were under computer control: two mass flow controllers (Type 825, Datametrics, Wilmington,

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MA) and two sets of Peltier modules (Melcor Inc., Trenton, NJ). The flow controllers provided coarse and fine control of CO_2 levels in the gas mixture flowing through the leaf chamber. One set of Peltier modules controlled the temperature of a humidifying column, while the other set controlled the chamber temperature. The environmental variables that could be regulated with computer control of these components were air temperature, humidity, and C_a.[2] Physiological variables, including C_i, carbon assimilation, leaf temperature, conductance, and the VPD between leaf and air could also be regulated indirectly with this gas exchange system. In the experiments described here, actual and target values of variables under computer control were compared every 60 s. A proportional control algorithm (2) was used to set command voltages to components under computer control to maintain actual values close to target values. Data were recorded to computer disk every 12 min, and periods of rhythms were derived from sine waves fitted to the data (IGOR, WaveMetrics, Lake Oswego, OR).

The plants used in these experiments were red kidney bean, *Phaseolus vulgaris* L. (var Blue Lake Bush 274, W. Atlee Burpee Co., Warminster, PA), grown either in greenhouses under natural lighting near the time of the autumn equinox or in growth chambers under combined fluorescent-incandescent lighting. Plants grown in the greenhouse were maintained at temperatures of 28°C (day) and 20°C (night). Plants grown in growth chambers were exposed to the temperature and photoperiod conditions described in the legends accompanying each figure. The plants were grown individually in pots containing one liter of vermiculite and perlite (1:1 v/v). The plants were frequently super- and subirrigated with a nutrient solution and flushed with tap water weekly. Macronutrient concentrations in the nutrient solution were (mM): 2.5 NO_3^-, 0.5 NH_4^+, 3.0 K^+, 1.5 PO_4^{3-}, 1 Mg^{2+}, 1.3 Ca^{2+}, and 1 SO_4^{2-}. Micronutrients were present in the following concentrations (μM): 41 BO_3^{3-}, 9.7 Mn^{2+}, 1.5 Zn^{2+}, 0.3 Cu^{2+}, 0.15 Co^{2+}, 0.6 MoO_4^{2-}, and 12.5 Fe-EDTA. The center leaflets of fully expanded trifoliates were used for gas exchange measurements under a 1000 W high-intensity discharge multi-vapor lamp.

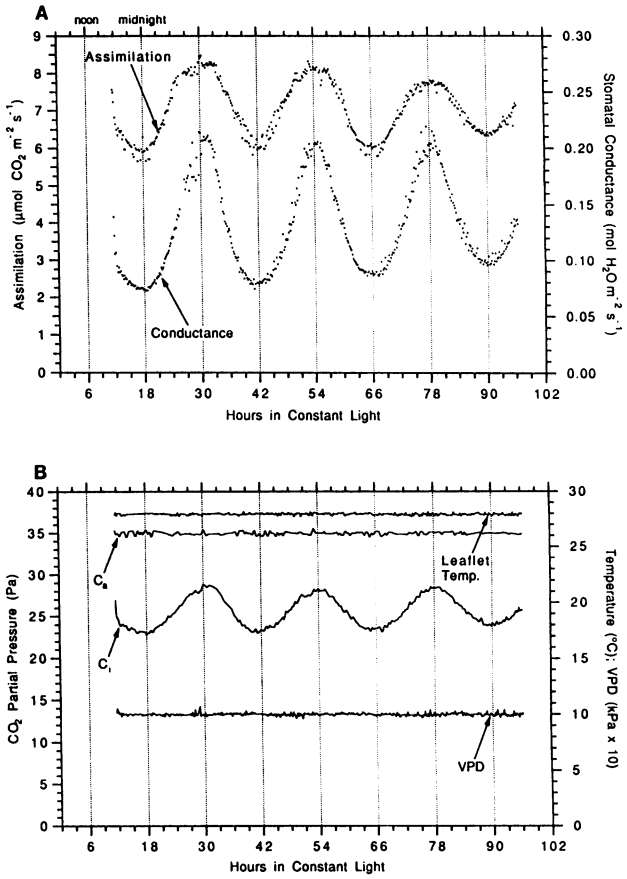

Figure 1. (A) Net carbon assimilation and stomatal conductance of a *P. vulgaris* leaflet exposed to constant light (200 μmol m^{-2} s^{-1}) and constant ambient CO_2 (35 Pa). (B) This plant developed in a greenhouse under natural lighting before it was transferred to constant light. The C_a, leaflet temperature (28°C), and VPD (1 kPa) were held constant. Data were recorded every 12 min and the lines in B are drawn through points corresponding to those in A. The data are plotted against hours in constant light with 6 = subjective noon and 18 = subjective midnight. The data shown are for a single plant but are representative of data from five other plants.

RESULTS AND DISCUSSION

Net carbon assimilation and stomatal conductance oscillated repeatedly in *Phaseolus vulgaris* L. plants moved from a natural photoperiod to constant light (Fig. 1A). In this experiment, a *P. vulgaris* leaflet that developed under natural lighting in a greenhouse was exposed to constant C_a, leaflet temperature, and VPD under light of constant intensity (Fig. 1B). Assimilation and conductance exhibited clear and persistent rhythms with little damping in the amplitude of these rhythms after 3 d under constant conditions. The rhythms were closely coupled with each other, reaching maximum values near noon and minimum values near midnight. The amplitude of the stomatal rhythm was particularly large, with conductance more than doubling between midnight and noon. The amplitude of the rhythm in carbon assimilation was smaller but still significant, with approximately a 30% increase between midnight and noon.

When the ambient CO_2 partial pressure is held constant, as in Figure 1, both stomatal and nonstomatal processes can influence the rate of carbon assimilation. Under conditions of constant C_a, the rhythm in stomatal conductance may cause a strong rhythm in C_i (Fig. 1B). Oscillations in C_i can directly affect the rate of assimilation by varying the amount of CO_2 available for fixation by photosynthesis. The influence of stomatal conductance on C_i can be suppressed, however, by adjusting C_a to hold C_i constant. Under conditions of constant C_i, any rhythm in carbon assimilation must be caused by nonstomatal processes. Assimilation and conductance in *P. vulgaris* continued to oscillate (Fig. 2A) when the intercellular CO_2 partial pressure was held constant (Fig. 2B). This experiment demonstrated that the rhythm in carbon assimilation was not caused solely by stomatal effects on C_i

[2] Abbreviations: C_a, ambient CO_2 partial pressure (Pa); C_i, intercellular CO_2 partial pressure (Pa); VPD, vapor pressure difference (kPa).

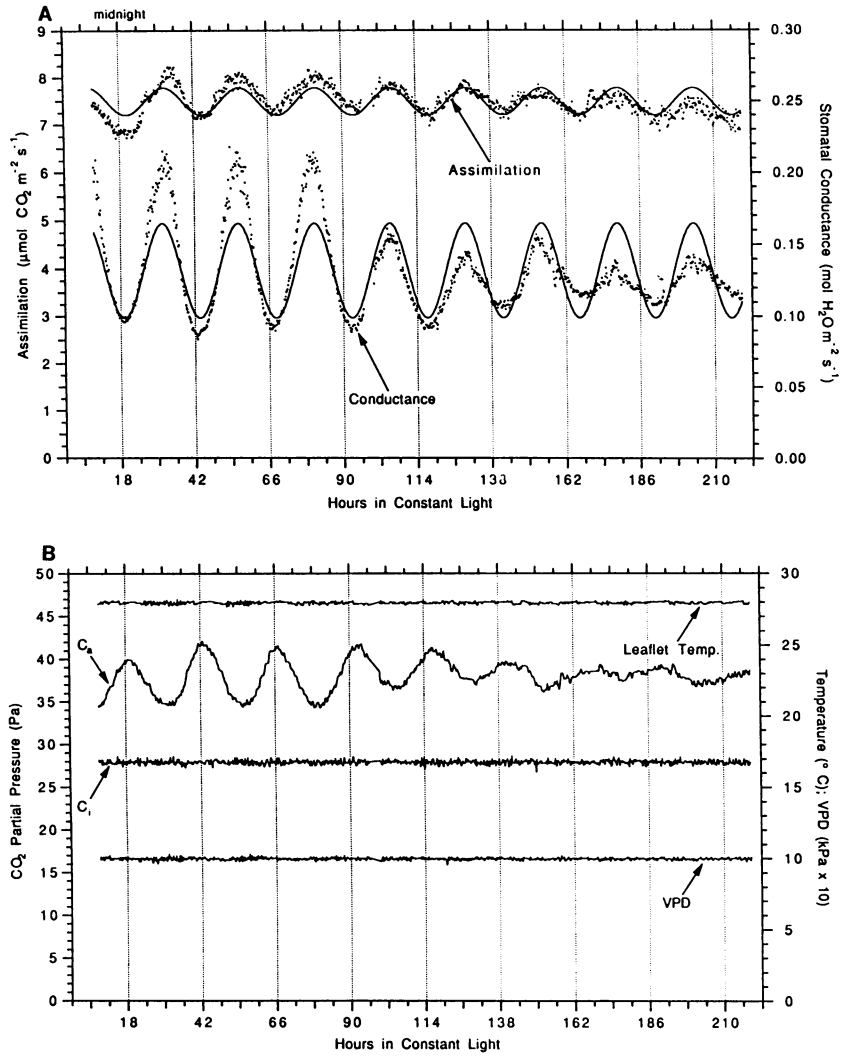

Figure 2. (A) Net carbon assimilation and stomatal conductance of a *P. vulgaris* leaflet exposed to constant light (200 μmol m^{-2} s^{-1}) and constant intercellular CO_2 (28 Pa). The recorded data are shown as points; sine waves fitted to the data are shown as solid lines. This plant developed in a growth chamber at a constant temperature of 28°C under a 12-h photoperiod (400 μmol m^{-2} s^{-1}) corresponding to h 0 to 12 on this graph. (B) The C_i was held constant by varying the C_a. The leaflet temperature (28°C) and VPD (1 kPa) were also held constant. The lines in B are drawn through points corresponding to those in A. The data are plotted against hours in constant light with 6 = subjective noon and 18 = subjective midnight. The data shown are from a single plant, but are representative of data from five other plants.

levels. Under these light and temperature conditions, the amplitude of the assimilation rhythm with constant C_i was approximately 50% of the amplitude with constant C_a.

When calculating the intercellular CO_2 partial pressure, one assumes that the degree of stomatal opening is approximately the same across a leaf. Variation in stomatal opening, or 'patchiness,' can occur in ABA-treated and water-stressed plants, producing spatial variation in C_i and photosynthetic activity across a leaf (8, 24, 26). The plants used in these experiments, however, were well-watered and unlikely to exhibit patchiness. To confirm this assumption, we assayed patchiness across leaflets maintained in constant light with a video-imaging technique (8). We found no significant patchiness across leaflets at noon or midnight, indicating that patchiness did not affect our measurement of C_i or cause the rhythm in assimilation (data not shown).

The amplitude of rhythms in conductance and assimilation damped under constant conditions, but these rhythms were clearly evident after more than a week in constant light (Fig. 2A). The rhythms in carbon assimilation and stomatal conductance remained in phase with each other over this long period of time during which C_i was held constant. Synchronization of assimilation and conductance indicated strong coupling between the rhythms, even when the rhythm in assimilation was the direct result of nonstomatal processes. Prolonged exposure to constant conditions also made clear that the period of these rhythms was slightly longer than 24 h; sine waves fitted to the data in Figure 2A had periods of 24.5 h. This is typical of circadian rhythms, which generally have free-running periods close to, but not exactly, 24 h in length.

The demonstration of a circadian rhythm in carbon assimilation with constant intercellular CO_2 levels is consistent with other studies on vascular plants indicating the involvement of nonstomatal processes in photosynthetic rhythms (7, 10, 19). Until this time, however, no one has documented a rhythm in carbon assimilation under conditions of constant C_i. Also, the conclusions of previous studies on this phenomenon were based on short-term experiments, generally 3 d or less in length, with sampling intervals of 3 h or longer. In this study, the rhythm was analyzed over a long period of time with frequent sampling, allowing accurate calculation of the free-running period of the rhythm in carbon assimilation. Furthermore, these experiments establish the phase relation-

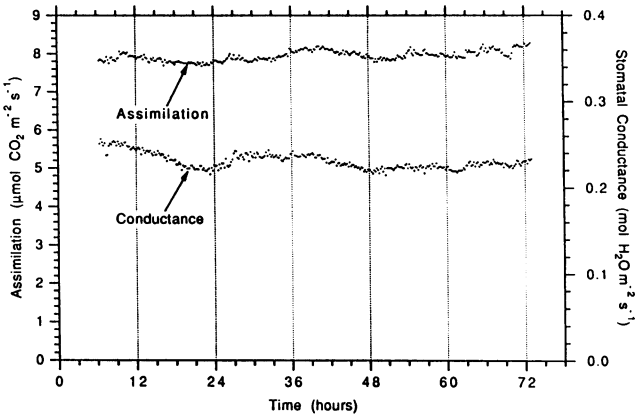

Figure 3. Net carbon assimilation and stomatal conductance of a *P. vulgaris* leaflet exposed to constant light (200 μmol m^{-2} s^{-1}) and constant intercellular CO_2 (28 Pa). This plant developed in a growth chamber under constant light (approximately 200 μmol m^{-2} s^{-1}) at a constant temperature of 28°C. The leaflet temperature (28°C) and VPD (1 kPa) were held constant during this experiment. The data shown are from a single plant but are representative of data from three other plants.

ship between rhythms in carbon assimilation and stomatal conductance, a relationship that was unclear in earlier studies using less frequent sampling intervals (10, 18, 21).

The leaflet described in Figure 2 developed in a growth chamber at constant temperature under a 12-h photoperiod with a light intensity of approximately 400 μmol m^{-2} s^{-1} during the photoperiod. When leaflets that developed in greenhouses under natural lighting were exposed to the same conditions with constant C_i, there was no difference in the expression of the rhythms in assimilation and conductance (data not shown). Apparently, a defined photoperiod in the absence of any temperature change was sufficient to entrain these rhythms. Also, it was the timing of the photoperiod that entrained these rhythms and not whether the photoperiod was presented as a square wave, as occurs in a growth chamber, or as a sinusoidal wave, as occurs under natural lighting in a greenhouse.

The damping of rhythms under constant conditions suggests that external time cues during growth, such as cycles of light and darkness, are necessary for expression of rhythms in assimilation and conductance. This suggestion was confirmed by studies with plants grown under constant light at a constant temperature. Carbon assimilation and stomatal conductance in plants grown under these conditions did not oscillate (Fig. 3), unlike plants grown under a cycle of light and darkness. This result demonstrated that circadian rhythms in photosynthesis do not occur spontaneously, but must be induced and coordinated by an external signal.

Experiments with plants grown in growth chambers at a constant temperature demonstrated that cycles of light and darkness were sufficient to entrain circadian rhythms (Fig. 2A). In natural environments, significant changes in temperature often accompany changes in light intensity. Temperature cycles can sometimes entrain circadian rhythms in the absence of any change in light intensity (23), although this phenomenon has not been demonstrated for rhythms in stomatal conductance and carbon assimilation. The possibility that temperature cycles might entrain these rhythms was investigated in plants grown under constant light with a 24-h cycle of high and low temperatures. When photosynthesis in these plants was measured under constant conditions, stomatal conductance oscillated but carbon assimilation did not (Fig. 4A). In this experiment, as in Figures 2 and 3, the intercellular CO_2 level was held constant so that the rhythm in stomatal conductance did not influence C_i. The rhythm in stomatal conductance was synchronized with the temperature cycle during growth so that maximum values corresponded to the middle of the high temperature period while minimum values corresponded to the middle of the low temperature period (Fig. 4B). The free-running period of the rhythm in stomatal conductance entrained by these conditions was comparable to that entrained by cycles of light and darkness. However, the rhythm in stomatal conductance damped more quickly in plants grown under a temperature cycle (Fig. 4A) than in plants grown under a cycle of light and darkness (Fig. 2A), suggesting that this rhythm was more strongly entrained by light than by temperature.

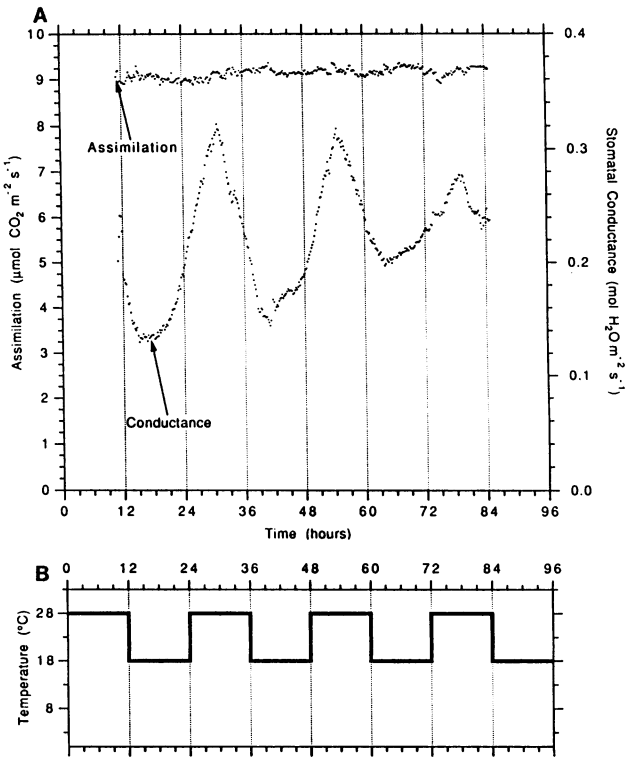

Figure 4. (A) Net carbon assimilation and stomatal conductance of a *P. vulgaris* leaflet exposed to constant light (200 μmol m^{-2} s^{-1}) and constant intercellular CO_2 (28 Pa) at a constant leaflet temperature (28°C). The VPD (1 kPA) was also held constant during this experiment. (B) This plant developed in a growth chamber under constant light (approximately 200 μmol m^{-2} s^{-1}) with a 24-h cycle cycle of high (28°C) and low (18°C) temperatures. The data are plotted against hours in constant light at a constant temperature. The data shown are for a single plant but are representative of experiments from three other plants.

In plants grown under cycles of light and darkness, rhythms in carbon assimilation and stomatal conductance were synchronized (Figs. 1A, 2A). This was not the case in plants grown under constant light with a temperature cycle, in which only a rhythm in stomatal conductance was induced (Fig. 4A). Possibly, the oscillator regulating stomatal conductance responds to both light and temperature while the oscillatory system regulating carbon assimilation is entrained only by cycles in light availability.

The persistence of a rhythm under constant conditions with a free-running period of approximately 24 h is the most significant feature of a circadian rhythm. Additional characteristics of a circadian rhythm are temperature compensation of the rhythm's period and sensitivity of the rhythm to a phase-shift with the appropriate stimulus. There is already evidence that circadian rhythms in stomatal conductance are temperature compensated (14) and can be phase-shifted by altering the photoperiod (16, 20). The rhythms in assimilation and conductance in *P. vulgaris* also satisfy these corollary criteria of circadian rhythms (manuscript in preparation, T.L.H.).

Gas exchange studies over the last several decades have provided a detailed description of photosynthesis in intact leaves. Most gas exchange studies, however, have recorded photosynthetic responses over periods of time too brief to reveal the effect of circadian rhythms on photosynthesis. The results presented here demonstrate that circadian rhythms have a significant influence on photosynthetic processes in *P. vulgaris*. In plants grown under natural conditions, circadian rhythms in stomatal conductance and carbon assimilation were coordinated with each other and the photoperiod so that, even in the absence of external time cues, maximum rates of photosynthesis occurred near noon and minimum values near midnight. Although the rhythm in carbon assimilation was closely coupled with the rhythm in stomatal conductance, nonstomatal processes were a significant component of the rhythm in carbon assimilation. Also, these rhythms varied in their sensitivity to environmental stimuli: cycles of light and darkness during growth entrained circadian rhythms in both stomatal conductance and carbon assimilation, but a temperature cycle under constant light induced only a rhythm in stomatal conductance.

Modification of photosynthetic responses by circadian rhythms may benefit plants by coordinating physiological activity with diurnal changes in light availability. The rhythmic opening and closing of stomata, for example, enhances the flow of CO_2 into a leaf during the day while minimizing water loss and evaporative cooling at night. Similarly, circadian rhythms in nonstomatal processes may reflect the partitioning of resources between photosynthetic activity during the day and nonphotosynthetic activities at night. A thorough description of photosynthesis must account for the potentially important role circadian rhythms play in regulating photosynthetic processes.

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