Diurnal Changes in Volume and Specific Tissue Weight of Crassulacean Acid Metabolism Plants

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ABSTRACT

The diurnal variations in volume and in specific weight were determined for green stems and leaves of Crassulaceae plants (CAM plants). Volume changes were measured by a water displacement method. Diurnal variations occurred in the volume of green CAM tissues. Their volume increased early in the light period reaching a maximum about mid-day, then the volume decreased to a minimum near midnight. The maximum volume increase each day was about 2.7% of the total volume. Control leaves of C3 and C4 plants exhibited reverse diurnal volume changes of 0.2 to 0.4%. The hypothesis is presented and supported that green CAM tissues should exhibit a diurnal increase in volume due to the increase of internal gas pressure from CO2 and O2 when their stomata are closed. Conversely, the volume should decrease when the gas pressure is decreased.

The second hypothesis presented and supported was that the specific weight (milligrams of dry weight per square centimeter of green surface area) of green CAM tissues should increase at night due to the net fixation of CO2. Green CAM tissues increased their specific weight at night in contrast to control C3 and C4 leaves which decreased their specific weight at night. With Kalanchoe daigremontiana leaves, the calculated increase in specific leaf weight at night based on estimates of carbohydrate available for net CO2 fixation was near 6% and the measured increase in specific leaf weight was 6%.

Diurnal measurements of CAM tissue water content were neither coincident nor reciprocal with their diurnal patterns of either volume or specific weight changes.

Characteristically green CAM tissues fix net quantities of CO2 at night, temporarily store the carbon in their large vacuoles as malic acid, and then the next day internally release the CO2 for photosynthesis. These temporal and spatial separations of activities associated with CAM are maintained by the primary use at night of their storage carbohydrate supply i.e., either starch or glucan or free hexoses (2), to synthesize PEP* for CO2 fixation rather than to translocate the carbohydrate as sucrose like many other plants. CAM tissues generally are succulent in the sense of their green cells having large vacuoles which may occupy near 95% of the total cell volume, and CAM plant stomata are open at night when net CO2 fixation occurs and closed during part of the day. The diurnal cycle of stomata activities are related to the water loss and gain of CAM tissues with a general tendency for water vapor transfer to increase at night when stomata are open and to decrease during stomatal closure in the day. Thus, CAM plants conserve water and tend to hydrate during the day; then the tissues transpire and tend to dehydrate at night.

Many of these characteristic diurnal aspects of CAM have been known for decades (8, 11). By combining these known features with recent research on CAM (6, 13), two hypotheses were developed which we have tested and are presented in this paper. First, we reasoned that the volume of green CAM tissues should increase during the day when stomata are closed due to the internal accumulation of gases such as CO2 and O2 from metabolism. Secondly, we reasoned that the specific weight of green CAM tissues should increase at night due to the net fixation of O2. We have partially tested these hypotheses by measuring the diurnal changes in volume and in the specific weight of green tissues from several CAM plants. These changes with green CAM tissues are compared to similar diurnal measurements made with leaves of a C3 and a C4 plant.

MATERIALS AND METHODS

Plant Material. Cereus sp. and Sansevieria trifasciata Prain were the CAM plants used to determine diurnal changes in plant volume. Corn (Zea mays var. Rugosa) and Rhoeo spathacea were used as the C3 and C4 plant controls.

Kalanchoe daigremontiana (Hammet and Perrier) plants were used to determine diurnal changes in the specific leaf weight of a CAM plant. Corn and Chinese cabbage (Brassica rapa L., Pekinensis group) were used as the C3 and C4 plant controls.

All plants were grown in a greenhouse, except corn which was grown outdoors. Greenhouse plants were watered daily (Brassica and Rhoeo) or every other day (CAM plants), and the corn was watered daily. All plants were fertilized with a commercial 15-15-15 fertilizer weekly. In general, we used fully expanded and younger leaves in the canopy.

To insure CAM, plants were transferred to plant growth chambers at least 2 weeks prior to use in any experiments. Experimental plants were placed under growth chambers with day and night temperatures of 30°C and 16°C, respectively. Plants were exposed to cycles of 15-h light and 9-h dark periods. At 0.5 m above the chamber floor, the fluorescent and incandescent lighting gave an illumination intensity of 200 μE m⁻² s⁻¹ (400–700 nm) which was near the canopy top for most experimental plants. Relative humidities of 70% in the light period and 90% in the dark period were maintained in the growth chambers.

Plant Volume Measurements. Plant volume was measured by a water displacement method. According to the shape of the leaves or of the succulent stems, H2O displacement volume measurements were made using different containers which matched the leaves or stems.

For measuring the volume of succulent stems, pots of experi-
mental plants were covered with cardboard at the stem base and sealed with tape. A stem was inserted upside down to a constant position on the stem into a cylinder containing H2O. The change in H2O level was recorded by using a 0.1-ml pipette which had the H2O siphoned into it. The pipette was calibrated with the cylinder volume so we could determine the H2O displacement volume.

For measuring the volume of leaves, a flat, custom built plastic box containing H2O was used which matched each leaf type. The outlet of the plastic box was connected to a small rubber tube and to a 10-μl pipette. Similar to the stem measurements, the pipette was calibrated with changes in the box volume so we could measure the H2O displacement volume.

Specific Leaf Weight Measurements. Measurements of specific leaf weight were made by a half-leaf sampling method (9).

In one set of experiments, the petioles or bases of the leaves were steamed to reduce or stop translocation, while in a second set of experiments no heat was applied. In the steam treatment experiments, the leaf petioles or bases were wrapped with cheesecloth and boiling water added. The heated cheesecloth was attached for 3 to 4 min to kill the phloem cells to stop the translocation of assimilates.

The experimental leaves were selected (24 leaves for K. daigremontiana and eight leaves for B. rapa and corn) from two to four pots of plants. To facilitate the detection of the selected leaves, they were each tagged with a small piece of tape bearing a number to show the time order. After steaming, the left or right half of each lamina was removed with a razor. These leaf halves were sampled and these, eight or 24, half leaves constituted the zero time controls for the subsequent sampling times.

Thirty to 40 leaf discs were bored from a half-leaf section with a known diameter cork borer at timed intervals. After boring, the discs were put into an oven at about 95°C and the residual of the half leaf was kept moist in paper towels. At each sample time, the matching half leaf was excised and bored at the same positions as the control half leaf using the residual half as a pattern.

After a successive series of diurnal sampling, the oven temperature was increased to 105°C and kept for 24 h. The samples were then cooled in a desiccator and weighed. The dry weights were determined to a constant value using additional drying as necessary. Specific leaf weight was calculated by dividing the leaf dry weight (mg) by the leaf surface area (cm²).

Measurement of Water Content. The diurnal variation in CAM tissue water content was measured by obtaining the fresh weight of samples collected at timed intervals. The tissue samples were dried to a constant dry weight and the % water content calculated.

Stomatal Diffusive Resistance. The diffusive resistance of the lower leaf surface of S. trifasciata was measured with a diffusive resistance porometer manufactured by Lambda Instruments Company, Inc. Diffusive resistance was measured at timed points and at the same leaf positions throughout 24 h. Temperature corrections were applied on the basis of measurements using the thermometer built into the sensor probe.

Measurement of Leaf Titratable Acidity. At the time of taking the specific leaf weight samples (K. daigremontiana) or at timed intervals (S. trifasciata), about 1 g leaf samples were removed, weighed, and frozen. The frozen tissues were ground in a tissue grinder containing about 4.0 ml of CO₂-free distilled H₂O and then boiled for 10 min in a H₂O bath. The homogenate was transferred to a centrifuge tube, cooled to room temperature, and centrifuged at 7190g for 10 min; the supernatant was collected in a 50-ml cylinder; the pellet was extracted and centrifuged one additional time; and the final volume was brought to 50 ml. These extracts were titrated to pH 8.3 with 10 mm NaOH, and the acid contents of the tissue were expressed as meq of acid/100 g fresh weight.

RESULTS

Measurement of the Diurnal Changes in Plant Volume. These measurements were either with the entire stem of potted plants or with leaves attached to potted plants. All diurnal experiments were performed at least five times and the results were averaged and plotted in the figures.

The volume increased with both the green succulent stem of Cereus, and with the attached leaves of Sansevieria in the light (Figs. 1 and 2). The volume reached a maximum near 15 h (3 pm), then decreased continually until midnight to reach a minimum, which was followed by an increase late at night. The magnitude of the volume increases were as much as 2.7 to 2.8% of the total plant volume (Figs. 1 and 2).

On the contrary, the control plants, corn and Rhoeo leaves, decreased their volumes during the light period presumably due to the higher temperature and the low humidity which combine to accelerate transpiration. These plants increased their volume in the dark period, about 0.2 to 0.4%, presumably due to the lower temperature and the high humidity which combine to rehydrate the tissue (diurnal volume data for C₃ and C₄ plants are not shown).

Fig. 1. Diurnal volume changes in the green succulent stems of Cereus sp. The % increase in volume was calculated with 24 h taken as zero change. The stems were attached and rooted.

Fig. 2. Diurnal variations in leaf volume and in leaf water content with S. trifasciata. The leaves were attached to a growing plant and volume changes were calculated as in Figure 1.
As already stated, these experiments were performed at least five times and simultaneously we began to measure other diurnal characteristics of CAM. In Figure 2, we have plotted the diurnal change in water content of Sansevieria leaves (these results will be discussed later), and Figure 3 shows the diurnal changes in titratable acidity and diffusive resistance for Sansevieria leaves along with the daily volume changes.

In true CAM fashion (5, 6), the leaf titratable acid content decreased after illumination to reach a minimum in the afternoon near 18 h (6 pm), increased in the darkness, and reached a maximum in the beginning of the light period (Fig. 3). Simultaneously upon illumination, the leaves showed an increase in diffusive resistance after about 1 h of light. Presumably, the stomata then were tightly shut, remaining so during the morning hours and early afternoon. Stomata began to open later in the afternoon and continued open in the dark, except for a transient increase in stomatal diffusive resistance after dark (near 22 h in Fig. 3).

The diurnal data on diffusive resistance and titratable acid are not coincident with the changes in plant volume, though we believe they are related. After illumination, when malic acid begins to be decarboxylated (the decline in acid content in Fig. 3), CO₂ increases inside of CAM tissues (3) as stomatal closure occurs; these events are followed by an increase in plant volume (Fig. 3). Later in the afternoon, when malic acid decarboxylation is nearly complete, stomata open and a plant volume decrease follows. Thus, the diurnal changes in plant volume are closely related to the acid content and to the condition of the stomatal aperture particularly during the light period.

**Measurement of the Diurnal Changes in Specific Leaf Weight.** Experiments were performed to compare the difference in specific leaf weight between CAM, C₃, and C₄ plant leaves. The experiments were divided into two types: in one, the leaf petioles or bases were heated with boiling H₂O; and in the other, these were not heated. The results are given in Figures 4 and 5. In Figure 4, corn and Chinese cabbage both decreased their specific leaf weight in the dark period probably due to respiration; corn decreased from 2.86 to 2.82 mg cm⁻² and Chinese cabbage decreased from 3.47 to 3.35 mg cm⁻² from 15 to 21 h. But K. daigremontiana increased its specific leaf weight from 9.66 to 10.11 mg cm⁻² over the same period (Fig. 4).

In Figure 5, the specific leaf weight decrease in the dark period was more than that in Figure 4 since the leaf petioles or bases were not heated, so more material was removed from leaves to the other parts of the plant. The specific leaf weights of corn and Chinese cabbage were decreased from 2.77 and 3.26 mg cm⁻² to 2.52 and 3.04 mg cm⁻², respectively, over the dark period. K. daigremontiana not only did not decrease its specific leaf weight but it also had an increase from 10.32 to 10.65 mg cm⁻² over the dark period. These results indicate that the assimilation of CO₂ by K. daigremontiana at night was more than the consumption of metabolites due to respiration or other metabolism plus any translocation in the dark period which is not true for the C₃ or the C₄ plant (Fig. 5). Note that, as expected, in the light all of these green tissues show an increase in specific leaf weight (Figs. 4 and 5).

**Diurnal Changes in the Specific Leaf Weight and Titratable Acid of Kalanchoë Leaves.** To gain further insight into this phenomenon, particularly the night phase, we compared the diurnal changes in specific leaf weight and titratable acid using the same K. daigremontiana plants but with steamed petioles to reduce or stop translocation. The results are shown in Figure 6. Titratable acidity fluctuated in a manner characteristic of CAM plants, it reached a maximum in the beginning of the light period and a minimum in the afternoon (5, 6, 11). The increase in titratable acid at night and the decrease early each day both are quite

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**Fig. 3.** Composite graph of the diurnal variations in titratable acid, diffusive resistance (or stomatal resistance), and the change in volume of attached S. trifasciata leaves.

**Fig. 4.** Diurnal variations in the specific leaf weight of a CAM, a C₃, and a C₄ plant. In these experiments, leaf petioles or bases were steamed but remained attached to the whole plant.

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In the course of this study, we determined the diurnal variation in water content of CAM tissues (Figs. 2 and 5, top). The data shown for these two CAM tissues exhibit the same tendencies, namely, to dehydrate at night when the stomata are open and to rehydrate during the day when stomata are closed. These tendencies have been recognized for many years with CAM tissues (7, 8). We wish to consider the relationships of diurnal variations in water content to diurnal changes in volume and specific leaf weight. If the diurnal variations in water content of CAM tissues cause cells to swell during the day and to shrink at night, our studies would be confounded by these variations.

However, as shown in Figure 2, the diurnal change in volume in CAM tissues is not coincident nor reciprocal with water content inasmuch as hydration occurs throughout the day and dehydration throughout the night while the volume peaks and declines independently. Or if one plots the water content data in Figure 5 on Figure 2, the light peak volume increase is near the peak water content, but no coincidence occurs at night. Similarly, if one compares the diurnal water content data (Figs. 2 and 5, top) with the diurnal specific leaf weight data (Figs. 4, top and 5, top), the data are neither coincident nor reciprocal for these CAM tissues. The amplitude of the water content changes are 0.6 and 0.67% (Figs. 2 and 5), which is less than either the volume or specific leaf weight changes.

We conclude that in CAM tissues the diurnal variations in water content do influence volumes and thereby may influence specific leaf weights, but these are not consistent relationships of the same magnitude over a day. Indeed, MacDougall (7, 8) noted reverse changes in the volumes of C₃ plant tissues which also correlated with water content. We earlier noted similar volume changes for Rhoeo leaves and corn leaves and yet simultaneously recorded increases in their specific leaf weight (Figs. 4 and 5). Thus, we propose that the specific tissue weight increases at night in CAM plants (Figs. 4–6) are not due to dehydration nor do the volume changes (Figs. 1–3) follow the diurnal patterns of water content. Rather, as will now be discussed, the metabolic production and consumption of CO₂ and O₂ during CAM are the dominant components of both volume and specific tissue weight changes.

DISCUSSION

Diurnal Changes in the Volume of Green CAM Tissues. We wish to propose that the diurnal variations in green CAM tissue volumes appear to be controlled by two major internal tissue components: CO₂ and O₂. Though these components have not been measured in this work, each of these components has a characteristic diurnal pattern of production and/or loss which is well documented in the literature (3, 5, 6, 12, 13).

The decline in acid content upon illumination proceeds upon the increase in stomatal resistance and occurs prior to the increase in plant volume (Fig. 3). Inasmuch as the CO₂ released in the light in CAM plants has its origin in malate, it might be expected that a relationship exists between acid content and the stomatal resistance. These ideas are in close agreement with the data obtained by Cockburn et al. (3); e.g. when stomata are open, the internal CO₂ of CAM tissue is low, but when stomata are closed the internal CO₂ is high, indeed reaching values as high as 2.5% (3 to 4.2% (12).

Because stomata are closed in part of the light period (from near 8 to 18 h in Fig. 3), the internal concentrations of O₂ of CAM plants also should be high. O₂ evolution from photosynthesis within this closed leaf system may raise the tissue O₂ concentrations rather rapidly. Indeed, the O₂ concentration rises to as much as 41.5% (12) within CAM tissues during this period of stomatal closure in the light.

Collectively, the data on green CAM tissues indicate that the increase in CAM plant volumes (Figs. 1–3) are related to the
stomatal resistance and the resultant restriction of gas exchange. The stomatal resistance is high during the light period and gas exchange is low which results in an increase in the partial pressures of CO\textsubscript{2} and O\textsubscript{2}. Therefore, green CAM tissues swell, or their volume increases. Later in the afternoon, stomata begin to open, resulting in a much-reduced resistance to gas transfer then, and throughout the dark period, stomata are open and gases transfer resistances are lower. Thus the tissue volumes decrease except late in the dark period. The reason for the increase in volume during the late dark period (Fig. 3) is unknown. Note that all of our plant material was watered well. Green tissues of control C\textsubscript{3} and C\textsubscript{4} plants exhibit reverse diurnal volume changes involving 0.2 to 0.4% levels.

After completing these studies on diurnal changes in volume, we reexamined the literature on diurnal volume changes in plants, particularly in trees, which is a widely recognized phenomena. D. T. MacDougal, over the general period of 1924 to 1936 (7, 8), reported the daily variations in the diameter and volume of tree trunks, herbaceous stems, and fleshy fruits and, broadly summarized, found them to shrink during the day and to swell at night. In marked contrast, he found a reverse diurnal variation in the diameter of the tree cactus, Carnegiea gigantea, and of flattened joints of Opuntia sp. (both are CAM tissues) (7). He noted that the closure of stomatal slits during the day and the loss of tissue acid was associated with the swelling of the tree cactus during the day (8). He attributed the increased daytime swelling of tree cactus to: (a) the narrowing of stomatal slits with a lessened H\textsubscript{2}O loss, and (b) the decrease in acidity which caused the hydration capacity of the cortex to increase. Both would result in cells swelling. The reverse explanation was used for the night shrinkage.

Indeed, our data on diurnal volume changes such as those in Figures 1 or 2 or 3 will superimpose on the diurnal changes in Figure 43 of Reference 8 with a remarkable coincidence, though we do not know the photoperiod in Figure 43. Figure 43 (8) is a dendrographic trace of the diurnal variation in the trunk thickness of the tree cactus over 8 d. Thus, our studies on the diurnal volume changes in green CAM tissue volumes confirm MacDougal's work with other CAM plants. We calculated from his data that: (a) based on the water content of tree cactus, the diurnal volume change would be ~0.7% of the total volume (8); or (b) based on stem diameter data and assuming a cylinder shape, the diurnal volume change would be ~1% of the total stem volume (7), our measured changes are greater, ~2.7% (Figs. 1–3). However, our explanation of the daytime swelling is different from MacDougal's in that we propose that the gas pressure increases from CO\textsubscript{2} and O\textsubscript{2} cause the daytime swelling.

Diurnal Changes in the Specific Leaf Weight of Green CAM Tissues. Specific leaf weight is widely employed in crop physiology studies and has been found to be related to net photosynthesis in soybean (4) and alfalfa (10). However, Watanabe and Tabuchi (14) observed no correlation between net photosynthesis of soybean cultivars and specific leaf weight. Environment, especially light intensity, greatly influences the specific leaf weight in many species (1). Leaves developed in dim light are thinner and have a lower specific leaf weight than leaves developed in bright light. In our experiments, we found that leaves growing at different layers in the plant canopy had a different specific leaf weight and that plants at different growth stages had different specific leaf weights.
Thus, it was important to maintain plants in the same environmental conditions prior to use in an experiment and to select comparable leaves. We could not find literature data on the diurnal variations of specific leaf or tissue weight with CAM plants.

Inasmuch as specific leaf weight is closely related to photosynthesis, respiration, and translocation, in this study we utilized steam treatment as a tool to reduce or stop translocation (cf. Figs. 4 and 5). When the leaf petioles were heated, the assimilates of photosynthesis could not be transported readily from leaves to other plant parts. This was particularly true with CAM plants, and in the dark the accumulation of titratable acid occurred rapidly and the specific leaf weight increased concurrently (Fig. 6).

Control C₄ and C₃ plants, corn and Chinese cabbage, decrease their specific leaf weight in the dark period due to respiration and increased through the light period (Fig. 4). When the leaf petioles were not heated, the control plants decreased their specific leaf weight in the dark period even more, presumably due to translocation (Fig. 5). At the same time, the CAM plant had an increase in specific leaf weight due to the accumulation of malic acid which was more than metabolic consumption and any translocation in the dark period (Fig. 5).

Two time periods are particularly interesting in the diurnal data on specific leaf weight with CAM tissues because related data are available on the internal CO₂ (3, 12) and O₂ (12) concentrations. These time periods are the light period after stomata close (24-7 h in Figs. 4-6) and the period at darkness (14-15 h in Figs. 4-6) when stomata close temporarily (Fig. 3). At both of these time periods, the specific leaf weight of CAM tissues exhibit sharp decreases (Figs. 4-6). Our explanation for these sharp decreases is that, at both time periods, internally the tissues have elevated levels of CO₂ (3, 12), and in the light period their O₂ level is elevated (12). In measuring specific leaf weight, both gases, CO₂ and O₂ would be lost during the drying process; therefore, the sharp decreases in specific leaf weight at both time periods. At subsequent time periods of measuring specific leaf weight, the CO₂ has been or is being fixed and O₂ is evolved so the specific leaf weights increase.

Collectively, these experimental results are consistent with the thesis that in darkness the specific leaf weight increase in CAM plants is caused by the increase in internal acid due to net CO₂ fixation. Indeed, one can make a more quantitative evaluation of the night data in Figure 6. We previously noted (2, 5) that CAM plants make a massive metabolic investment in that 15% to 20% of their total dry matter turns over every day in operating CAM. For example, 20% of their total constituency turns over as carbohydrates each night to synthesize PEP for CO₂ fixation (2, 5). Based on this value, if one calculates the increased weight in fixing CO₂ to form oxaloacetic acid from PEP, about a 30% increase in weight will occur. Thirty percent of 20% is about a 6% net increase in weight. In Figure 6, the specific leaf weight increases from 9.63 to 10.25, or an increase of about 6% in specific leaf weight.

Note, we do not think these diurnal changes as we are interpreting them relate to net growth of the leaves or tissues, although growth is certainly occurring as can be seen in most of the figures. These diurnal changes in volume and specific leaf weight would be superimposed on net growth curves, just as they are superimposed on diurnal water content changes.

In conclusion, we believe the results of this work support both of the proposed hypotheses. First, that green CAM tissues do increase their volume during the day during stomatal closure, and that this is likely due to the increased gas pressure from the internally generated CO₂ and O₂. And second, that the specific leaf weights of green CAM tissues do increase at night due to the net fixation of CO₂. This conclusion is quantitatively supported by the data in Figure 6 and the calculations above, both of which show an expected and measured increase of about 6% in the specific weight of green CAM tissues. In addition, the literature data on internal CO₂ and O₂ (3, 12) changes throughout a day support both conclusions.

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