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Dark CO_2-Fixation and Diurnal Malic Acid Fluctuations in the Submerged-Aquatic *Isoetes storkii*

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Summary. In the leaves (but not corms) of the submerged aquatic *Isoetes storkii* malic acid concentration fluctuated from 22 μeg g FW^{-1} in the evening to 171 μeg g FW^{-1} in the morning. Associated with this was a change in titratable acidity of 152 μeg g FW^{-1} between morning and evening. ^{14}C carbon was fixed in both the light and the dark, though the amount of carbon fixed in the light was more than that fixed in the dark. Autoradiographs show 88% of $^{14}CO_2$ fixed in the dark is recovered after 1 h, in malic acid and the remainder in one other unidentified product, whereas these two products contain less than 15% of the ^{14}C fixed after 1 h exposure to $^{14}CO_2$ in the light. It is suggested that CAM metabolism in this aquatic species may be related to the low availability of CO_2 for photosynthesis during the day in its aquatic environment and that this metabolic pathway may prove common in the genus *Isoetes*.

Introduction

Dark-fixation of CO_2 is widespread in plants, but only in CAM (Crassulacean Acid Metabolism) plants does it substantially contribute to the total carbon gain (Kluge and Ting 1978). In CAM plants CO_2 is fixed at night, through β-carboxylation of phosphoenolpyruvate, into malic acid which is stored in the vacuole. During the day malic acid is removed from the vacuole, decarboxylated and the CO_2 released is fixed by the Calvin cycle. In terrestrial CAM plants this pathway allows Calvin type photosynthesis to proceed during the day with stomates closed, thereby conserving water.

Recent evidence indicates that CAM also occurs in the submerged aquatic *Isoetes howellii* Engelmann (Keeley 1981). This plant is capable of CO_2-fixation in the dark and in the light. The major dark fixation product is malic acid and when submerged, malic acid concentration fluctuates from ca. 30 μequivalents per g fresh weight in the late afternoon to ca. 150 μeg gm FW^{-1} in the early morning. $^{14}CO_2$ fixed in the dark is transferred from malic acid to carbohydrates in light.

The purpose of this study was to determine if the same photosynthetic pathway was present in another *Isoetes* species from a very different aquatic situation.

The family Isoetaceae is a group of "lower" vascular plants distributed world-wide in aquatic (freshwater) or moist habitats (Pfeiffer 1922). *Isoetes* species all posses quill-like leaves arising from a corm. Each leaf has four longitudinal air canals, separated from one another by septa and surrounded by an outside wall of green tissue. *Isoetes howellii* is distributed throughout western North America and is often classified as amphibious in that it normally occurs in seasonally aquatic environments. The experiments discussed above were done on plants from vernal pools at sea level in southern California, where *I. howellii* is submerged during the winter and spring but persists only as a dormant corm during the summer drought. The experiments presented here deal with *Isoetes storkii*, Palmer, a species morphologically similar to *I. howellii*, but distributed in an aquatic habitat quite unlike vernal pools in which the latter species is found. *Isoetes storkii* is a submerged aquatic in lakes of volcanic origin above 2,500 m in Costa Rica.

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Materials and Methods

Isoetes storkii was transferred from Lagunna del Poas (2,600 m), a lake in the crater of Volcan Poas, Costa Rica, to greenhouse aquaria in Los Angeles. Water level was maintained above the tips of the leaves.

Leaves were sampled at 0700 h and 1,500 h (PDT) for titratable acidity and malic acid concentrations. Leaves were washed in distilled water, blotted dry, weighed, ground with distilled water, filtered through cheesecloth, and centrifuged at low speed. Titratable acidity was determined on an aliquot of the supernatent, titrating to the phenolphthalein endpoint with 0.01 N NaOH. The remainder of the supernatent was deproteinized with 1 N perchloric acid and assayed enzymatically for malic acid (Bergmeyer 1974, pp. 1585–1589).

Relative $^{14}CO_2$-fixation in the light and dark was measured on leaves at 1,500 h. Intact leaves were washed in distilled water, blotted dry, weighed, and placed in four 50 ml round bottom flasks filled with 5 mM $KHCO_3^-$ acidified with HCl to pH 6. Two flasks were covered with aluminum foil and two flasks were exposed to incandescent light at 1,200 μE M^{-2} s^{-1} (PAR) in a 25 ± 1° C waterbath. After 15 min all flasks were injected with 28 μCi $NaH^{14}CO_3$ (10 mCi/mM) and incubated for exactly 1 h. CO_2-fixation was terminated by adding boiling 80% ethanol to each flask. After acidifing to remove unfixed ^{14}C, end products were extracted by boiling for 45 min and then counted on a scintillation counter. Labelled products were separated with 2-way paper chromatography using phenol in one direction and butanol-proprionic acid in the other direction as described by Pedersen et al. (1966). Autoradiographs were developed from the chromatograms.

Results

Leaves of *Isoetes storkii* showed a highly significant ($P < 0.001$ with t-test) 6 fold increase in titratable acidity and malic acid concentration overnight (Fig. 1).

$^{14}CO_2$ fixation experiments were conducted to determine if nighttime malic acid accumulation resulted from dark fixation

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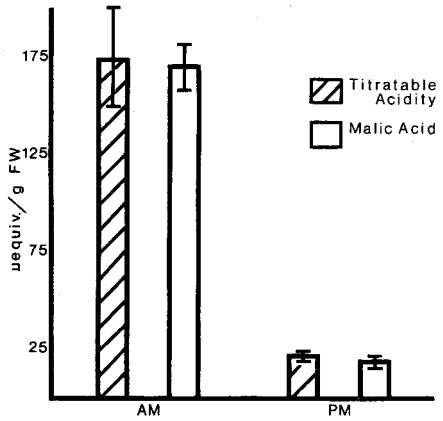

Fig. 1. Titratable acidity and malic acid concentration in leaves of *Isoetes storkii* at 0,700 h and 1,500 h. Each bar is the mean of 4 replicates and the vertical lines are ±1 SD

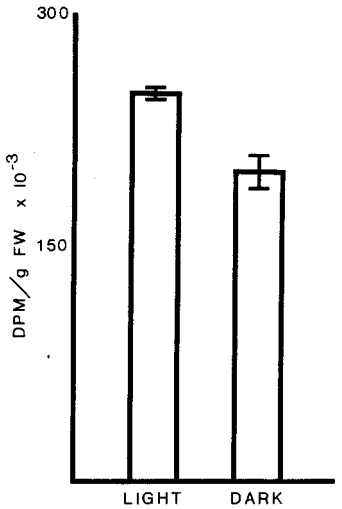

Fig. 2. Relative $^{14}CO_2$-fixation by leaves of *Isoetes storkii* in the light and dark. Each bar represents the mean of 2 replicates. Vertical lines indicate ±1 SD

of CO_2. Figure 2 shows that *I. storkii* is capable of fixing CO_2 in the dark and the light, and relative to the latter, dark fixation is substantial.

Autoradiographs of dark-fixation products indicated only two products labelled. These were eluted and counted. Eighty-eight percent of the label was in one product which was shown to be malic acid by the enzymatic assay (methods section). The lesser product was not identified. The autoradiographs of the light fixation products showed label in various amino acids and carbohydrates but less than 15% in malic acid.

Discussion

Isoetes storkii shows all of the same Crassulacean Acid Metabolism characteristics observed in *I. howellii* (Keeley 1981). The metabolic similarities between these *Isoetes* species and terrestrial CAM plants are striking. Perhaps the major difference between *Isoetes* and terrestrial CAM plants lies in differences in stomatal behaviour. Prototype CAM plants restrict CO_2 uptake during the light due to stomatal closure (Kluge and Ting 1978). Both *Isoetes howellii* and *Isoetes storkii* have stomata but these are apparently nonfunctional (Sculthorpe 1967; Keeley 1981) and these species are capable of CO_2 uptake in the light. That stomata are not involved in diurnal acid metabolism is further illustrated by the presence of diurnal malic acid fluctuations in species of *Isoetes* which lack stomata (Keeley in press).

The relative contribution of light vs. dark CO_2-fixation is unknown. Gas-exchange studies with *I. howellii* (Keeley and Bowes unpublished data) indicate that, under high CO_2 concentrations, net CO_2-uptake rates are greater in the light than in the dark. The adaptive significance of CAM in *I. howellii* is perhaps related to the fact that vernal pools often have marked diurnal changes in availability of free-CO_2. These pools are heavily vegetated, shallow and clear and the photosynthetic depletion of CO_2 may occur before noon, particularly on warm days. However, such an explanation seems less likely to apply to *I. storkii*. This species is found in high elevation oligotrophic lakes which are sparsely vegetated and, although data are not available, it seems unlikely that these habitats would have large diurnal changes in CO_2 availability. Such lakes are characterized, however, by very low levels of total CO_2 (Hutchinson 1957).

Because of the contrast in habitats between *Isoetes howellii* and *I. storkii* (relative to other *Isoetes* species) it seems likely that this CO_2 fixation pathway will prove to be common in the genus *Isoetes*.

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