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# $C_4$ Acid Fixation in Photosynthesis of the Submerged Aquatic *Eleocharis acicularis* (L.) R. & S.

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ABSTRACT

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*Eleocharis acicularis* (L.) R. & S. is a widely distributed submerged aquatic macrophyte. In southern California, it codominates shallow seasonal pools with the aquatic CAM plant *Isoetes howellii* Engelm. Like the latter species, *E. acicularis* is apparently restricted to uptake of free-$CO_2$; bicarbonate uptake is negligible, as indicated by poor carbon fixation at pH 7 and higher oxygen evolution at pH 5 than at pH 8 across a range of free-$CO_2$ concentrations. Unlike *Isoetes*, *Eleocharis* does not have CAM photosynthesis, however it does have the capacity for a low level of carbon fixation in the dark. $^{14}C$ tracer studies in the light reveal $C_4$ acids constitute 40–50% of the initial labeled products, however the leaves lack Kranz anatomy. It is suggested that in seasonal pools, photosynthesis by *E. acicularis* is highest in the early morning when ambient free-$CO_2$ levels are maximal. Such sites are typically overcast in the early morning and like *I. howellii*, *E. acicularis* is light saturated at relatively low irradiance levels. Throughout much of the day, free-$CO_2$ concentrations are limited and oxygen concentrations are high. PEP carboxylase mediated carbon fixation may be of selective value under such daytime conditions.

## INTRODUCTION

*Eleocharis acicularis* (L.) R. & S. is a submerged aquatic rhizomatous sedge widely distributed throughout the world. Across its range, *E. acicularis* occurs in a variety of aquatic habitats, from shallow seasonal pools to lakes. In seasonal pools it is exposed to ambient conditions of marked diurnal fluctuations in pH and inorganic carbon concentrations, in contrast to the more stable environment of deeper permanent bodies of water (Keeley and Morton, 1982). This species is distributed in lakes exhibiting a relatively wide range of water chemistry. In northern Europe, it is equally frequent in lakes which are circumneutral as in alkaline lakes (Hutchinson, 1975). In North America this species is found in soft-water acidic lakes ranging from pH 3.9–6.9 (Yan et al., 1985),

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as well as in alkaline lakes (Moyle, 1945), and the same has been noted for this species in high elevation tropical lakes in the Andes of South America (J.E. Keeley, personal observations, 1982–1985).

Such a remarkable range of conditions is not generally noted for aquatic taxa and thus this species is interesting for its ability to exist under such diverse conditions. It is noteworthy that throughout its range, *E. acicularis* coexists in both seasonal pools and oligotrophic lakes with species of *Isoetes*, a genus noted for its unusual photosynthetic pathway of CAM. In many of the habitats *E. acicularis* and an *Isoetes* species may codominate a site (Keeley and Morton, 1982; Keeley et al., 1983). Although it appears that *E. acicularis* does not possess CAM photosynthesis (Keeley and Morton, 1982), little else is known about its photosynthetic characteristics. The purpose of this study was to examine quantitative and qualitative aspects of photosynthesis in this species from seasonal pools in southern California.

## METHODS

### *Plant material*

Samples of *E. acicularis* were collected in April 1983 from a vernal pool on Mesa de Colorado, Santa Rosa Plateau (elevation 610 m), Riverside Co., CA, U.S.A. All plants were growing under submerged conditions when transplanted with soil to aquaria housed in environmental growth chambers and to outdoor experimental pools at Occidental College. Aquaria were filled to the tips of leaves with deionized water and maintained on a diurnal pattern of 12 h dark at 13°C and 12 h light at 18°C, which approximated springtime submerged conditions at the study site (Morton, 1984). These artificial pools had complements of other associated species native to the natural habitat. Water chemistry followed a similar diurnal pattern in pH, $CO_2$, and $O_2$, typical of field conditions. Leaf cross-sections were prepared for light microscopy using standard techniques of alcohol dehydration and embedding in parafin.

### *Quantitative carbon uptake*

Photosynthetic rates were estimated by measuring oxygen evolution with a Yellow Springs Instruments (YSI)-53 oxygen monitor and a YSI-5331 Clark-type polarographic sensor. Samples were harvested between 10:00 and 13:00 h, washed with deionized water, and cut into 2-cm lengths to fit the sample chamber. Oxygen evolution in the light (1000 $\mu$mol PAR $m^{-2}$ $s^{-1}$) was determined at pH 5 (25 mM Na-Citrate) and pH 8 (25 mM Tris-HCl) across a range of free-$CO_2$ concentrations (see Van et al., 1976). Ten millilitres of freshly prepared $CO_2$-free buffer and 0.1 g of leaf material were added to the sample chamber and equilibrated with stirring in room light at 25°C for 5 min. Ex-

periments were initiated by injecting 0.2 ml of appropriate $NaHCO_3$ solution and recording $O_2$ evolution for 10 min.

A comparison of carbon uptake rates in the light and dark was estimated by measuring $^{14}C$ incorporation across the pH range and inorganic carbon level encountered under natural conditions (Morton, 1984). Leaves were harvested at either 08:00 h (a.m.) or 14:00 h (p.m.), cut into 3-cm segments, weighed, tied into bundles with fine thread, and suspended in 25-ml serum bottles containing 20.0 ml of 25 mM buffer (Na-phosphate: pH 6, pH 7; Tris-HCl: pH 8, pH 9) freshly made with $CO_2$-free distilled water ($dH_2O$). Bottles were stoppered and equilibrated in the light (1000 $\mu$mol PAR $m^{-2}$ $s^{-1}$) or dark for 15 min at 25°C. Mixing was provided by a stirring bar and glass beads were included to eliminate air spaces. Each experiment began by removing a 0.5-ml aliquot of buffer and injecting 0.5 ml of 24 mM $NaH^{14}CO_3$ for a final concentration of 0.6 mM $NaHCO_3$ and 0.5 $\mu$Ci $ml^{-1}$. After 10 min, the sample was dropped into boiling 80% (v/v) methanol for 5 min, homogenized, and centrifuged at 9200 *g* for 30 min. The pellet was washed once with 80% methanol, centrifuged, washed again with $dH_2O$, and centrifuged. The supernatants from each step were combined then evaporated to dryness at 105°C, resuspended in $dH_2O$, centrifuged, and counted on a liquid scintillation counter.

*Qualitative carbon incorporation patterns*

The initial products of carboxylation were determined with steady-state $^{14}C$ tracer studies. Leaves were harvested as previously described. Each bundle was dropped into 25-ml serum bottles containing 11.5 ml of 10.0 mM MES-NaOH (pH 5.5). Samples were equilibrated in the light or dark for 2 h at 25°C. Experiments were initiated by removing 1.0 ml of buffer, then injecting 1.0 ml of 1.0 mM $NaH^{14}CO_3$ with 25 $\mu$Ci $ml^{-1}$ and incubating samples for 1,5, 30, or 60 s (light), or 10, 60, 300, or 600 s (dark). Experiments were terminated by adding samples to boiling 80% methanol. Samples were prepared as described in the previous section. A sample of the final suspension was taken for liquid scintillation counting and a sample was used for separation of photosynthetic products by two-dimensional thin layer electrophoresis and chromatography as described by Schurmann (1969).

*Enzyme assays*

Ribulose-1,5-biphosphate carboxylase (RuBPcase) and phosphoenolpyruvate carboxylase (PEPcase) activities were assayed at 25°C using $^{14}C$ fixation techniques and liquid scintillation counting of acid stable products. RuBPcase was assayed in the active form as described by Lorimer et al. (1977) and PEP-

case was assayed by the procedure of Van et al. (1976). Experiments were initiated by addition of substrate and terminated by the addition of 6 M HCl.

Chlorophyll was determined by the procedure of Arnon (1949).

## RESULTS

The photosynthetic light response curve for *E. acicularis* shows net $O_2$ evolution below 10 $\mu$mol PAR $m^{-2}$ $s^{-1}$ (Fig. 1). Photosynthesis was apparently light saturated at around 300 $\mu$mol PAR $m^{-2}$ $s^{-1}$.

Oxygen evolution rates across a range of free-$CO_2$ levels, at low and high pH, suggest that *E. acicularis* is unable to utilize $HCO_3^-$ (Fig. 2). Photosynthetic rates are clearly higher at pH 5 regardless of the free-$CO_2$ level, despite the fact that at pH 8 this represents substantially higher total inorganic carbon conditions; free-$CO_2$ comprised $>95\%$ of the total inorganic carbon pool at pH 5, but $<2\%$ at pH 8. At pH 5, rates increased exponentially up to 0.1 mM free-$CO_2$ and linearly above that concentration.

A comparison of carbon uptake, measured by $^{14}C$ incorporation, in the light and in the dark is shown in Fig. 3. Although there was detectable carbon uptake in the dark, it was more than an order of magnitude lower than in the light. In both the light and the dark, there was substantially less incorporation above pH 7, further indicating a lack of $HCO_3^-$ uptake. Also, in both the light and the dark there were marked differences in carbon uptake in response to time of harvesting. In the light, carbon fixation rates were greatest in the samples collected in the morning and run at pH 6, whereas in the dark, fixation rates were highest for samples collected in the afternoon and run at pH 7.

It is obvious that the rates of carbon uptake measured by $^{14}C$ incorporation

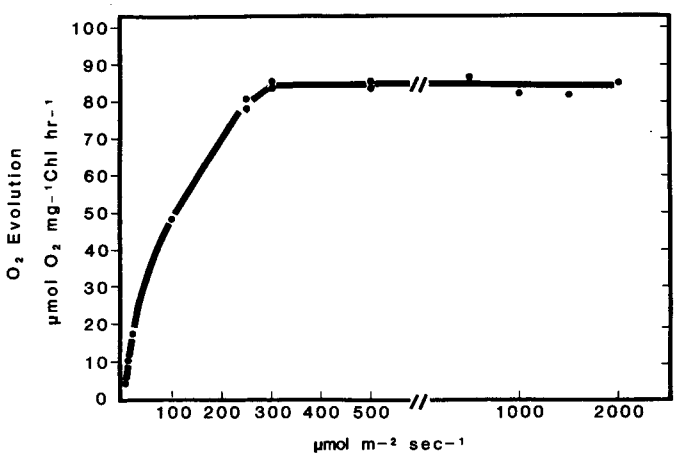


Fig. 1. Oxygen evolution rates in response to PAR irradiance levels at 0.9 mM free-$CO_2$, pH 6 and 25°C.

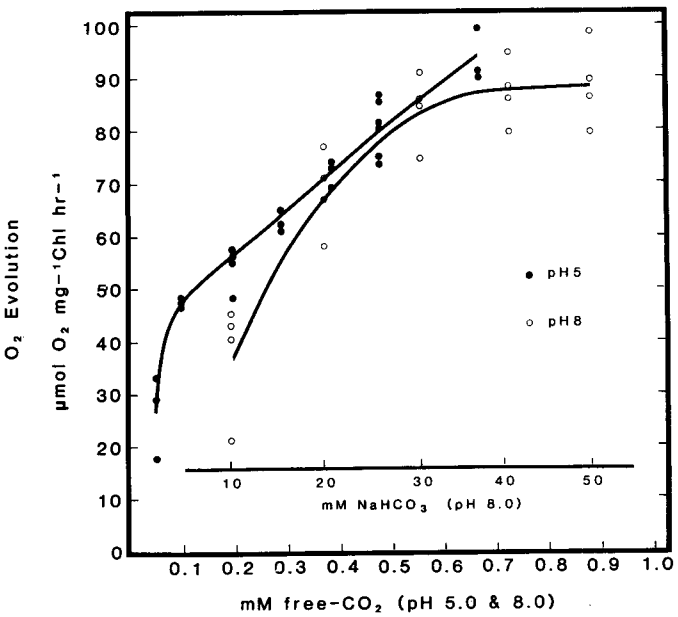


Fig. 2. Oxygen evolution rates in response to free-$CO_2$ concentrations at pH 5 and pH 8 in the light (1000 $\mu$mol PAR $m^{-2}$ $s^{-1}$) (inset scale shows inorganic carbon level required for given free-$CO_2$ concentration at pH 8.0).

(Fig. 3) were much lower than the rates of oxygen evolution measured with the oxygen electrode (Figs. 1 and 2). Several factors could account for these differences. For example, in the $^{14}C$ experiments leaves were tied in bundles to facilitate rapid removal, and thus circulation around the leaves would have been far less than for leaves floating freely in the oxygen electrode apparatus. In addition the degree of mixing generated by the stirring bar in the oxygen electrode apparatus was substantially greater; in the $^{14}C$ experiments glass beads (added to eliminate air space) greatly slowed the stirring bar. Finally, as lacunae act as reservoirs for $CO_2$, there may be a lag time in the actual fixation of the $^{14}CO_2$. In light of the fact that the pools occupied by *E. acicularis* are relatively stagnant, the absolute rate of photosynthesis under field conditions is probably closer to the lower values given by the $^{14}C$ technique. Regardless of the absolute rates, the relative conclusions drawn from these experiments should hold.

The initial products of $^{14}C$ incorporation in the light were largely divided between phosphoglycerate (3-PGA) and the organic acids malate and aspartate (Table 1). With longer incubation, proportionately less label was in all of these acids. In the dark, two-thirds of the label was fixed into malate and the remainder into aspartate (Table 2). The proportion of label in malate re-

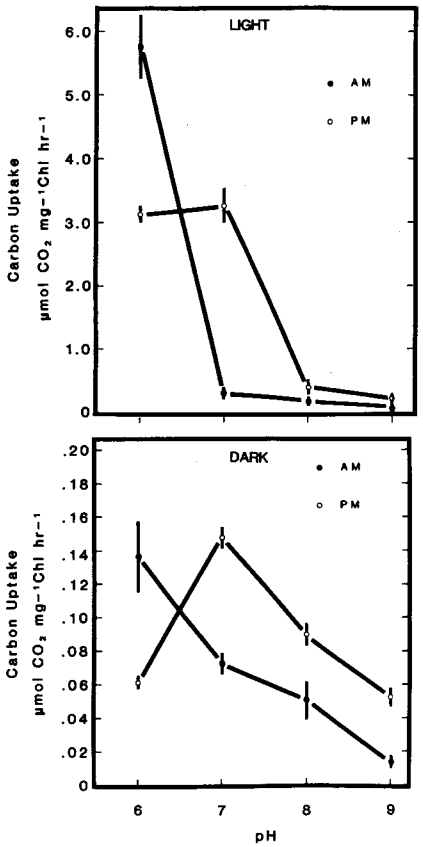


Fig. 3. $^{14}C$ incorporation in response to pH in the light (1000 $\mu$mol PAR $m^{-2}$ $s^{-1}$) and dark, and in the morning (08:00 h) and afternoon (14:00 h) at 0.6 mM $NaH^{14}CO_3$ at 25 °C ($n=2, \pm 1$ SD). pH = 6.0, 7.0, 8.0 and 9.0.

mained stable for up to 10 min incubation, whereas it decreased in aspartate, suggesting some turnover of that product.

Activities for the two carboxylating enzymes, RuBP carboxylase and PEP carboxylase, were assayed on submerged plants and plants exposed to the atmosphere for more than 2 weeks (Fig. 4). RuBP carboxylase had several times greater activity than PEP carboxylase and activity increased significantly upon emergence; the ratio of RuBPcase : PEPcase increased from 6.5 for submerged plants to 11.4 for emerged plants.

Examination of leaf cross-sections showed that the leaves of *E. acicularis* had five large lacunae which occupied approximately 75% of the cross-sectional area. Chloroplasts were abundant in the epidermis and single mesophyll cell layer. No evidence of Kranz anatomy was observed.

TABLE 1

Light fixation products of *Eleocharis acicularis* with steady-state $^{14}C$ labelling at 1000 μmol PAR $m^{-2}\,s^{-1}$, 25 °C, and 1 mM $NaH^{14}CO_3$, pH 5.5 ($n=3$). Recovery ranged from 55–75% of total label loaded onto the plate and standard deviation of the mean was less than 10% of the mean in all cases

| Fraction | Percentage of $^{14}C$ label in soluble fraction | | | |
|---|---|---|---|---|
| | Time (s) | | | |
| | 1 | 5 | 30 | 60 |
| Phosphoglycerate | 46.4 | 53.8 | 39.8 | 35.5 |
| Malate | 32.4 | 36.6 | 15.1 | 21.8 |
| Aspartate | 10.5 | 6.0 | 11.3 | 1.6 |
| Glucose-6-phosphate | 0 | 0 | 8.6 | 12.6 |
| Unidentified | 10.7 | 3.6 | 25.2 | 28.5 |

TABLE 2

Dark fixation products of *Eleocharis acicularis* with steady-state $^{14}C$ labeling at 25 °C, and 1 mM $NaH^{14}CO_3$ ($n=3$). Recovery and variance of the data were as reported in Table 1

| Fraction | Percentage of $^{14}C$ label in soluble fraction | | | |
|---|---|---|---|---|
| | Time (s) | | | |
| | 10<br>$n=$ 1 | 60<br>1 | 300<br>2 | 600<br>6 |
| Malate | 64.9 | 70.4 | 68.4 | 65.2 |
| Aspartate | 33.7 | 29.1 | 24.4 | 18.3 |
| Unidentified | 1.4 | 0.5 | 7.2 | 16.5 |

## DISCUSSION

In the shallow seasonal pools occupied by *E. acicularis* in southern California, there are marked diurnal changes in pool chemistry. Peak free-$CO_2$ levels in the water occur in the early morning and are usually depleted by noon. These sites are typically overcast in the early morning (before 09:00 h) and thus it is not surprising that photosynthetic rates of *E. acicularis* would be high at relatively low irradiance levels (Fig. 1). We predict that throughout much of the day, photosynthesis would be limited by availability of inorganic carbon as this aquatic macrophyte is apparently dependent to a large degree upon free-$CO_2$; the drop in $^{14}C$ incorporation at pH 8 and the lower $O_2$ evolution at pH 8, relative to pH 5, suggest no capacity for bicarbonate uptake (Figs. 2 and 3).

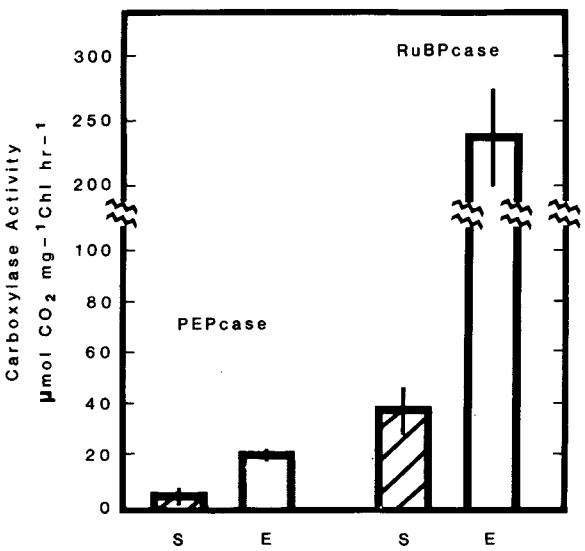


Fig. 4. Activity of phosphoenolpyruvate carboxylase (PEPcase) and ribulose bisphosphate carboxylase (RuBPcase) in *E. acicularis* leaves grown submerged (S) ($n=28$) and emerged (E) for more than 2 weeks ($n=12, \pm 1$ SD). Chlorophyll = 1.25 mg $g^{-1}$ fresh weight for both submerged and emerged plants.

Although this experiment is a reasonably good measure of the extent of bicarbonate use (see Van et al. 1976), this interpretation is complicated in the present study by the apparent inhibition of photosynthesis at pH 8.

With respect to its low light saturation and apparent lack of $HCO_3^-$ uptake, *E. acicularis* is similar to *Isoetes howellii* Engelm., a species with which it coexits (Keeley and Bowes, 1982; Keeley, 1983). *Isoetes howellii*, however, has substantial carbon uptake in the dark through Crassulacean Acid Metabolism. Dark $CO_2$ uptake by *E. acicularis* is of little quantitative significance (Fig. 3), but there is substantial $C_4$ acid fixation in the light (Table 1).

In this latter respect, *E. acicularis* has certain similarities to the subtropical aquatic macrophyte *Hydrilla verticillata* (L.f.) Royle (Holaday and Bowes, 1980; Salvucci and Bowes, 1983). This latter species has the capacity for carbon incorporation in the light through RuBP carboxylase as well as PEP carboxylase. It lacks Kranz anatomy and both carboxylases are present in the same cell (Bowes and Salvucci, 1984). Under summer conditions of long days and high temperatures, PEP carboxylase activities exceed RuBP carboxylase activities and the bulk of the carbon is initially fixed into organic acids.

Further detailed studies would be needed to determine if the $C_4$ acid fixation observed for *Eleocharis* represents the same $C_4$ pathway as found in *Hydrilla*. In *E. acicularis*, the $C_4$ acid fixation is not quite as active, at least under springtime conditions used in this study, as observed for *Hydrilla* under summer conditions. It is not known whether the activity of this pathway would increase

under longer days and higher temperatures; however, in nature this species would not be exposed to such conditions as it survives summer drought as a dormant rhizome.

Some level of $C_4$ acid fixation is also known from other submerged aquatic macrophytes, e.g. *Egeria densa* Planch. (Browse et al., 1980), *Elodea canadensis* Michx. (DeGroote and Kennedy, 1977) and *Orcuttia californica* Vasey (J.E. Keeley, unpublished data, 1986); however, as with *E. acicularis*, the degree to which these represent $C_4$ type photosynthesis is unclear.

J. Brown (personal communication, 1988) notes that $C_4$ acid fixation reported for *Egeria densa* (Browse et al., 1980) resulted from carbon starvation prior to the experiments, and under high carbon levels, fixation is through the $C_3$ pathway. In the field, *E. acicularis* is exposed to high $CO_2$ levels in the early morning and ambient free-$CO_2$ is depleted by mid-day. An intriguing question for future studies, is whether or not the pathway of carbon incorporation varies through the day with changes in water chemistry.

In summary, we predict photosynthesis in *E. acicularis* would be highest in the early morning when free-$CO_2$ is available in the pools. As the ambient free-$CO_2$ levels drop during the morning, photosynthesis should be limited due to its apparent inability to utilize $HCO_3^-$. During the morning, concomitant with a reduction in carbon dioxide, there is an increase in oxygen concentrations in the pool (Keeley and Busch, 1984). Such conditions should favor the utilization of PEP carboxylase mediated carbon fixation due to the photorespiratory effect of oxygen on RuBP carboxylase. These characteristics are interesting in light of the very wide distribution of *E. acicularis*. The inability to utilize bicarbonate would not be expected in a species found in alkaline lakes as noted for this species (Moyle, 1945; Hutchinson, 1975). Also the role of daytime $C_4$ acid fixation in softwater oligotrophic lakes, where oxygen saturation may not present problems for photosynthesis, is not readily apparent. Future studies however will need to determine whether or not *E. acicularis* shows similar photosynthetic characteristics across the range of environments in which it is distributed.

## ACKNOWLEDGMENTS

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## NOTE ADDED IN PROOF

After this manuscript was accepted Ueno et al. (1988, Proc. Natl. Acad. Sci., 85: 675) reported an opposite pattern for *Eleocharis vivipara* where submerged foliage is $C_3$ and aerial foliage is $C_4$. Also, the prediction of higher a.m. photo-

synthesis made in the present article has been borne out by field studies (Keeley and Sandquist, Ecology, in press).

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