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Photosynthetic carbon assimilation by *Crassula helmsii*

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Abstract Photosynthesis of *Crassula helmsii*, an amphibious aquatic macrophyte weed species, has been measured with respect to pH and irradiance. *C. helmsii* shows a marked diel fluctuation in titratable acidity, which can be accounted for by changing levels of malic acid. *C. helmsii* is unable to use HCO_3^- for photosynthesis and exhibits generally low photosynthetic rates when CO_2 is not limiting. The photon flux density at which the onset of light saturation of photosynthesis is reached (E_K) is low for aquatic macrophytes. Some advantages conferred on *C. helmsii* by the possession of crassulacean acid metabolism are an extension of the period of assimilation of dissolved inorganic carbon, resulting in a reduction in the limitation imposed on photosynthesis in aquatic environments by a very high CO_2 diffusion resistance.

Key words Aquatic macrophyte · Crassulacean acid metabolism · Nuclear magnetic resonance

Introduction

Crassula helmsii (T. Kirk) Cockayne is an amphibious aquatic macrophyte native to Australasia which is becoming a problem in lakes and other waterbodies in the British Isles (Dawson and Warman 1987). It is extremely competitive and tends to dominate the entire habitat once it has become established, to the detriment of native species. At present there are approximately 450 sites where *C. helmsii* occurs in the United Kingdom (F.H. Dawson, personal communication), and the doubling rate for new

occurrences is approximately 2 years (Dawson 1988). The ecology and growth requirements of this species in the United Kingdom are well documented (Dawson and Warman 1987; Kirby 1964 1965; Swale and Belcher 1982), but there is little available information on photosynthetic characteristics or on the extent of crassulacean acid metabolism (CAM).

Diurnal acid fluctuation in aquatic plants occurs mainly within the genus *Isoetes* (Keeley and Morton 1982; Bowes 1985) and is less widespread in other genera, having been reported only in the following species: *Crassula aquatica* (L.) Schönl., *Littorella uniflora* (L.) Ascherson (Keeley 1982; Keeley and Morton 1982), *Lilaeopsis lacustris* Hill and *Vallisneria spiralis* Graeb. (Webb et al. 1988). The ecological advantage to an aquatic plant possessing CAM has been suggested to be the enhancement of the acquisition of dissolved inorganic carbon (DIC) in carbon-limited environments (Keeley and Morton 1982; Madsen 1987). Further explanations have also been put forward (Farmer and Spence 1987; Richardson et al. 1984; Webb et al. 1988) which tend to support the lack of evidence for a common environmental trigger for the expression of CAM.

The possession of facultative CAM in aquatic plants is probably more broadly linked to competitive advantage. In the case of the isoetid life-form the advantage is clearer than in other groups. Although there is probably less competition in typical isoetid habitats (except some North American vernal pools: Keeley 1982), CAM may help in allowing the uptake and effective utilisation of CO_2 from the root zone over the majority of a 24-h day (Boston et al. 1987; Madsen 1987), thus alleviating the inherently low availability of DIC in the water column.

CAM in *C. helmsii* probably confers a competitive advantage in mesotrophic and eutrophic conditions. The ability to assimilate CO_2 during periods of the day when competitors are unable to photosynthesise, even though the rate of assimilation is not as high as that of some competitors, may help in allowing dominance to occur rapidly. Complete dominance has occurred within 18 months of the initial introduction in some sites (J.R.

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Newman, personal observation). This study attempts to partially identify some of the factors associated with photosynthetic carbon assimilation which contribute to the aggressive nature of this invasive aquatic plant.

Materials and methods

Origin of plant material and growth conditions

The material was obtained from the Royal Botanic Gardens, Kew (reference numbers 013-77-06059 and 497-77-00355, originally from Tasmania) through the Dundee University Botanic Gardens. The plants were multiplied by rooting 5-cm shoot segments in a mixture of 10:1 river silt and John Innes Number 1 compost. The plants were submerged to a depth of 30 cm in tap water amended with $1 \text{ mmol m}^{-3} \text{ FeCl}_3$, under ambient conditions of temperature and irradiance. Plant material used for experiments was collected from these stock plants and grown in a growth cabinet at 22.5°C , under a 11:13 h (day to night) regime at $320 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ photosynthetically available radiation (PAR, 400–700 nm). For Nuclear Magnetic Resonance (NMR) studies the material was grown at 15°C under a 12:12 h (day to night) regime at $150 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ PAR in 0.05 strength Hoaglands solution.

pH-drift experiments

The experimental theory of Spence and Maberly (1985) was used to explore the ability of *C. helmsii* to take up HCO_3^- from solution. In a solution of known alkalinity and DIC concentration the contribution of DIC species to the total DIC changes with pH in a closed system. Above pH 8.2 the concentration of CO_2 is close to zero, and the supply of DIC for photosynthesis must be derived exclusively from HCO_3^- (or CO_3^{2-}). The degree of utilisation of HCO_3^- can be expressed as a C_T/Alk value, where C_T (mol m^{-3}) is the final total dissolved inorganic carbon concentration and Alk (equivalents m^{-3}) is the final alkalinity value. C_T/Alk values in excess of 1.00 indicate a lack of HCO_3^- use, whereas C_T/Alk values of less than 1.00 indicate an ability to take up HCO_3^- , the ability increasing with decreasing values of C_T/Alk . Fresh plant material (1 g) was placed in a sealed glass bottle containing solutions of 1, 2, or 4 equivalents m^{-3} alkalinity (KHCO_3 in distilled water). The bottles were placed in a shallow water bath at 10, 15 or 20°C and illuminated for 24 h at $1000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The final pH was measured after 24 h and the (C_T) calculated.

Titrate acidity

Plant material was collected at dawn (0800 hours) and dusk (1900 hours), blotted dry and weighed. The material was ground up with liquid N_2 to give a fine powder. The powder was added to 25 cm^3 double-distilled water and heated to 60°C for 5 min. The pH of the water was measured after cooling to 25°C . The extract was titrated to pH 7.0 with KOH (50 mol m^{-3}), and the volume required was used to calculate the total $[\text{H}^+]$ in the extract. The difference in titrate acidity (TA) measured at 1900 and 0800 hours represents the amount of H^+ accumulated during the dark period. Measurements of TA during the light period were also carried out to monitor the reduction in TA during the day.

Organic acid analysis

Plant material was collected at the same time as for TA analysis and treated in the same way. The heated slurry was filtered (GF/C), made up to 50 cm^3 with double-distilled water and stored at 4°C . Boehringer-Mannheim test kits were used to assay for l-malic, isocitric and citric acid. NADP concentration was determined by spectrophotometric analysis.

Photosynthetic O_2 evolution

Photosynthetic rates of O_2 evolution were measured using a Rank Brothers dissolved oxygen electrode system, connected to a Servogor flatbed chart recorder. DIC was added as KHCO_3 or NaHCO_3 to provide a range of [DIC] from 0.01 mol m^{-3} to 10 mol m^{-3} . Illumination was provided from a 150 W quartz halide bulb. The relationship between photon flux density (PFD) and photosynthetic oxygen evolution rate was also measured using this apparatus, using neutral density filters to alter incident PFD on the thermostatically controlled water jacket of the apparatus. Photosynthesis was monitored for 15 min, sufficient for a steady rate to be achieved. Photo-inhibition was judged to have occurred if the measured net photosynthetic rate was less than the maximum rate achieved at lower incident PFDs.

Infrared gas analysis

One gram of freshly collected plant material was rinsed in distilled water and immersed in 15 cm^3 of $100 \text{ mmol m}^{-3} \text{ KHCO}_3$ buffered at pH 6.5 with $50 \text{ mol m}^{-3} 2\text{-}[N\text{-morpholino}]\text{-ethane sulphonic acid (MES)}$ in a 25 cm^3 test tube. The tube was placed in a thermostatically controlled water bath. Illumination was provided by a 150 W quartz halide bulb. The tube was connected into a sealed recirculating infrared gas analysis (IRGA) system and the concentration of CO_2 measured. The CO_2 compensation point was measured by providing continuous illumination of $500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and recording the $[\text{CO}_2]$ when no further decrease occurred. The diurnal fluctuation in $[\text{CO}_2]$ was measured by placing 1 g of plant material in the apparatus, illuminating at $500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ for 6 h and then placing in darkness for 24 h.

NMR spectroscopy

Plants were grown under the conditions described. Experiments used measurements of the fluctuation in ^{13}C levels at defined chemical shifts in the spectrum, associated with nocturnal accumulation and daytime decarboxylation of malic acid stored in the vacuole. Plant material (1 g fresh weight) was collected at the end of the dark period, cut into 4-cm lengths and placed in the bottom of a 10-mm-diameter NMR tube. The tissue was covered with D_2O . The ^{13}C spectrum was measured for 2 h to give a large enough signal to noise ratio. The tube was removed from the machine and illuminated at $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ at ambient room temperature (22°C) for 6 h. At the end of the light period the spectrum was measured for 2 h.

Results

Results of the pH-drift experiments are shown in Table 1. C_T/Alk values close to 1.00 represent an inability to remove HCO_3^- ions from the bathing solution (Spence and Maberly 1985). From data presented, it can be seen that *Crassula helmsii* is not able to use HCO_3^- under any of the experimental conditions tested. Furthermore, C_T/Alk values are independent of both temperature and alkalinity, indicating a restriction on carbon uptake ability imposed by a biochemical restriction rather than by any environmental parameter (Newman 1991). The results obtained here are in broad agreement with other data on aquatic CAM plants (Boston et al. 1987; Spence and Maberly 1985).

Results of measurements of TA throughout the light period are given in Fig. 1. The maximum value was always measured at 0800 hours, the end of the dark period,

Table 1 Final pH values, final total dissolved inorganic carbon concentration (C_T) values, and C_T /Alk ratios (where Alk is the final alkalinity value) for *Crassula helmsii* when exposed to solutions of increasing alkalinity at different temperatures. Values are means of three replicate determinations \pm standard error ($n=4$).

Temperature (°C)	Alk (equivalents m^{-3})	Final pH	Final C_T	C_T /Alk
10	1	8.30	1.00 ± 0.00	1.000
	2	8.45	1.99 ± 0.01	0.995
	4	8.69	3.98 ± 0.00	0.995
15	1	8.34	1.00 ± 0.00	1.000
	2	8.48	1.99 ± 0.00	0.995
	4	8.74	3.98 ± 0.01	0.996
20	1	8.36	1.00 ± 0.00	1.000
	2	8.53	1.99 ± 0.00	0.995
	4	8.72	3.98 ± 0.01	0.996

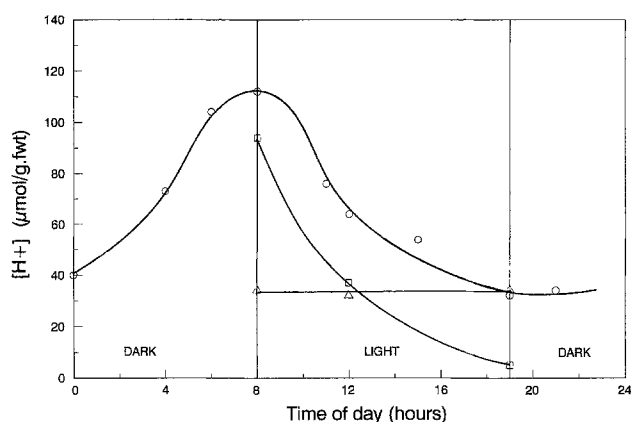


Fig. 1 Titratable acidity (\circ), malic acid content (\square) and isocitric acid content (\triangle) of *Crassula helmsii* measured during the day. Results are means of three determinations \pm SE. The light period extends from 0800 to 1930 hours (*fw* fresh weight)

and the minimum was always measured at 1900 hours, the end of the light period, indicating an overnight accumulation and a daytime reduction in TA characteristic of CAM. The measured values of malic acid account for almost all of the diurnal change in TA. It is assumed that malic acid is the only organic acid accumulated during the dark period by *C. helmsii*. The residual amounts of isocitric acid accounts for the remainder of the TA measured at 1900 hours. It is not clear if isocitrate levels are independent of malate accumulation or if isocitrate is an intermediate store of accumulated inorganic carbon.

Photosynthetic oxygen evolution occurs under conditions of zero external DIC and was measured at oxygen levels of less than 10% of air-saturation to minimise O_2 inhibition of ribulose biphosphate carboxylase oxygenase (RUBISCO) carboxylating activity. The results are shown in Fig. 2. At 1030 hours, 2.5 h after the start of the light period, the net rate of oxygen evolution is almost 80% of the DIC-saturated rate at pH 7.5 (Table 2). The net rate of oxygen evolution declines with the mea-

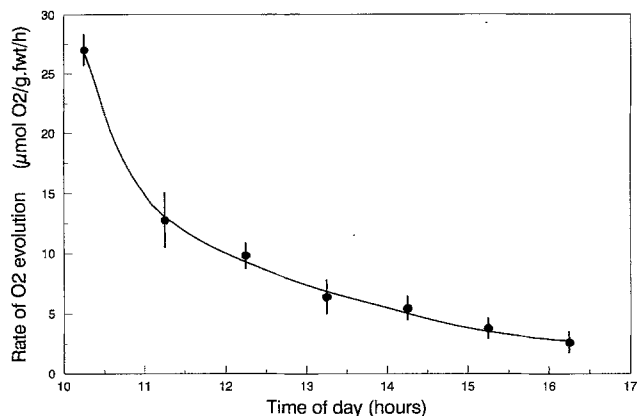


Fig. 2 The rate of photosynthetic oxygen evolution of *Crassula helmsii* in CO_2 - and O_2 -free media at pH 7.0, 15°C, during the day. Values are means of three determinations \pm SE. The light period extends from 0800 to 1930 hours (*fw* fresh weight)

Table 2 Dissolved inorganic carbon (DIC)-related photosynthetic parameters of *Crassula helmsii*. (V_{max} the maximum observed photosynthetic rate at 5 mol m^{-3} [DIC] and 1000 μmol photons $m^{-2} s^{-1}$, $K_{0.5}$ DIC value at which half the saturated O_2 evolution rate is achieved)

pH	V_{max} ($mmol O_2 g^{-1} Chl a h^{-1}$)		$K_{0.5}$ ($mol m^{-3}$)	
	10 °C	15 °C	10 °C	15 °C
6.5	28	37	0.11	0.20
7.0	14	35	0.21	0.38
7.5	27	30	0.65	0.52
8.0	8	17	0.29	0.56

sured TA during the day, and it is reasonable to assume that C_3 carboxylation and fixation of CO_2 proceeds at a rate which is dependent on the amount of malic acid remaining in the cell. If the rate of supply of CO_2 from decarboxylation of the dicarboxylic acids formed during the dark period is constant, then the observed rate of O_2 evolution would be $3.47 \mu mol g^{-1} fresh weight h^{-1}$. This theoretical rate is exceeded for the first 6 h of the light period, and we assume that the rate of decarboxylation proceeds faster earlier in the day, due to substrate abundance. The maintenance of photosynthesis during the initial 2 h of the light period, independent of external DIC, may reduce inter- and intraspecific competition for DIC by extending the time during which a limiting resource can be assimilated, without altering the external concentration of that limiting resource. Because *C. helmsii* is restricted to using CO_2 , the assimilation of CO_2 during this period may be a reflection of the inability to compete for DIC during later periods of the day when photosynthesis by other submerged macrophytes in mesotrophic environments have effectively removed all the free CO_2 from the water, leaving only HCO_3^- .

The results of photosynthetic rate versus PFD are presented in Fig. 3. The value of E_k (onset of light saturation; Kirk 1983) of $52.7 \mu mol$ photons $m^{-2} s^{-1}$, is ap-

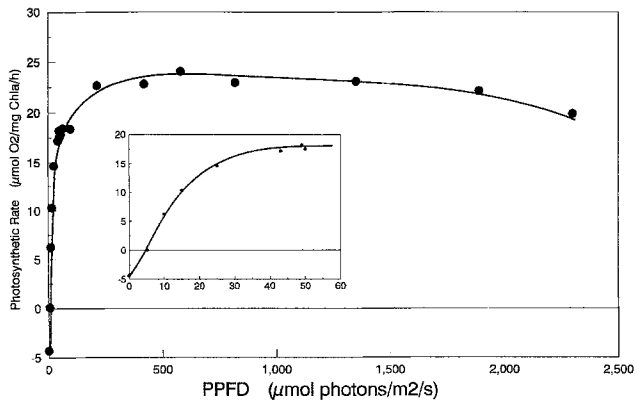


Fig. 3 The dependence of the net rate of photosynthetic O_2 evolution on incident photon flux density (PPFD) at 15°C , pH 7.5 and $[\text{DIC}]_{\text{external}}$ (DIC dissolved inorganic carbon) of 2 mol m^{-3} (NaHCO_3). The light period extends from 0800 to 1930 hours. Inset shows response between 0 and $50 \text{ } \mu\text{mol photons m}^{-2}\text{s}^{-1}$ for clarity on the same axis labelling

proximately 2.5% of full sunlight PFD at noon on a summer's day. This indicates an ability to achieve maximum photosynthetic rates at low light levels. This would be advantageous in that levels of malic acid, and hence the potential for the internal production of CO_2 , are high early in the morning when light levels are usually low. The light compensation point was interpolated from the graph of PFD versus photosynthetic oxygen evolution (Fig. 3), and was $4 \text{ } \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ for all samples tested. Photo-inhibition occurred above $1500 \text{ } \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Fig. 3), but is unlikely to be an important factor effecting photosynthesis of *C. helmsii*, and is not significant until incident PFD reaches $2400 \text{ } \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ over the 15 min of exposure tested. Maximum rates of photosynthesis are maintained over a range of incident PFDs from 250 to $1800 \text{ } \mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

The results of the effect of temperature and pH on the rate of photosynthetic oxygen evolution are given in Table 2. The maximum photosynthetic rate, V_{max} decreases with increasing pH, and $K_{0.5}$ (the DIC alue at which half the saturated O_2 evolution rate is achieved) increases with increasing pH. This indicates a dependence on $[\text{CO}_2]$ for photosynthesis in *C. helmsii*. The values given for pH 7.0 at 10°C are unusually low and indicate a restriction of the rate of DIC-saturated photosynthesis. The reason for this anomaly is unknown. Both V_{max} and $K_{0.5}$ increase with temperature.

The CO_2 compensation point of submerged-type material of *C. helmsii* is $82 \text{ cm}^{-3} \text{ m}^{-3}$ (Table 3). IRGA measurements showed uptake of CO_2 during the night period by submerged leaves, and no night-time uptake by aerial type material over the same time-scale (Table 3). Night-time CO_2 uptake did not exceed the amount of CO_2 released previously by respiration. CO_2 uptake started at midnight, 5 h after the start of the dark period, and continued until 0800 hours. The diurnal rhythm continued for a period of 48 h in complete darkness, although the

Table 3 Maximum observed rates ($\text{mmol CO}_2 \text{ g}^{-1} \text{ Chl } a \text{ h}^{-1}$) of CO_2 exchange of submerged and emergent type leaves of *Crassula helmsii*. The experimental conditions were 10°C , pH 6.5 in air equilibrated distilled water for submerged leaves, and 10°C in a humid atmosphere (derived from a small amount of distilled water at pH 6.5) for aerial type leaves. The values are the means of three determinations \pm standard errors. Note that crassulacean acid metabolism (CAM) was only detectable after the rate of CO_2 uptake (CAM) exceeded the loss of CO_2 from respiration. The values shown for CAM and dark respiration are net rates

Process	Leaf type	
	Submerged	Aerial
Dark respiration	8.02 ± 0.94	8.42 ± 0.40
Dark CO_2 uptake (CAM)	5.15 ± 0.60	0
Light CO_2 uptake (photosynthesis)	28.4 ± 2.07	29.60 ± 1.74
CO_2 compensation point ($\text{cm}^3 \text{ m}^{-3}$)	82	82

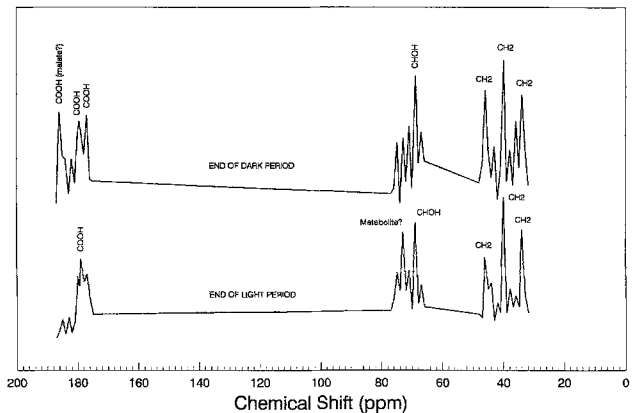


Fig. 4 Reproduction of the natural abundance ^{13}C Nuclear Magnetic Resonance spectra of *C. helmsii*. Peaks are marked and show possible identifications. The top spectrum was recorded between 0900 and 1100 hours, and the bottom spectrum between 1700 and 1900 hours, representing the end of the dark and light periods, respectively. The chemical shift is given in ppm

amplitude was reduced to about 50% during the period corresponding to the second light period. The apparent cessation of night-time uptake of CO_2 by emergent grown plant material is supported by work on other aquatic CAM plants (Keeley and Busch 1984).

The natural abundance ^{13}C NMR spectrum is shown in Fig. 4. There are two major differences between the morning and the evening spectra. The peaks at 184 ppm and 177 ppm in the morning spectrum, probably representing the COOH groups of malic acid, are not present in the evening spectrum. In comparison with other work on CAM plants using NMR, it is likely that the COOH peak at 177 ppm is the peak corresponding to the C-1 position on the malate molecule, and the peak at 184 ppm may correspond to the C-4 position. The chemical shift of these groups increases with increasing pH, and the lower equivalent chemical shift values quoted by Stidham et al.

Table 4 Data on crassulacean acid metabolism in submerged aquatic plants ranked according to Δ titratable acidity

Species	Δ Titratable acidity ($\mu\text{mol H}^+$ g^{-1} fresh weight)	Δ Malic acid ($\mu\text{mol mg}^{-1}$ Chl <i>a</i>)	Source
<i>Isoetes howellii</i> Engelmann	470	192	Keeley and Busch 1984
	245	109	Keeley 1989
<i>Isoetes orcuttii</i> A.A. Eaton	152	70	Keeley 1989
<i>Crassula aquatica</i> (L.) Schönl.	103	36	Keeley 1989
<i>Littorella uniflora</i> (L.) Aschers. var. <i>americana</i>	93	45	Keeley and Morton 1982
<i>Crassula helmsii</i> (T. Kirk) Cockayne	76	33	This study
<i>Isoetes kirkii</i>	67	–	Webb et al. 1988
<i>Valisneria spiralis</i> Graeb.	39	–	Webb et al. 1988
<i>Lilaeopsis lacustris</i> Hill	32	–	Webb et al. 1988
<i>Isoetes lacustris</i> L.	16	–	Madsen 1987
<i>Littorella uniflora</i> (L.) Aschers	12	–	Madsen 1987

(1983) correspond to lower vacuolar pH values measured in the *Kalanchoe* species used in that study. Other peaks remain almost unchanged, although the CHO groups have slightly higher chemical shifts in the evening spectrum, indicating a higher pH value at this time. The presence of isocitrate is indicated by other COOH, CHO and CH₂ groups, whose chemical shifts are the same in the morning and evening spectra, indicating relatively little change between the two assay times. The observed peaks are consistent with the presence of more than one organic acid within the tissue.

Discussion

Aquatic CAM plants generally exhibit low photosynthetic rates (Boston 1986; Sand-Jensen and Sondergaard 1978) and are not capable of HCO₃⁻ uptake from the external medium (Spence and Maberly 1985). Results presented here are in agreement with other data on aquatic CAM plants with respect to these parameters.

C. helmsii assimilates CO₂ at night, storing it as malic acid in the vacuole, and decarboxylates the malic acid during the day in addition to assimilating DIC by the classic C₃ pathway. The degree of CAM in *C. helmsii* in comparison with other aquatic CAM plants is presented in Table 4. The diel fluctuation in malic acid is similar to that in *C. aquatica*.

There is very little evidence from the distribution of *C. helmsii* in Britain that it is restricted to "isoetid-type" habitats, and given the low photosynthetic rate, is unlikely to be limited by the availability of DIC in such habitats. The dense monospecific mats of vegetation which this plant tends to form in shallow ponds may lead to extensive intraspecific competition for many resources.

The concept of CO₂ being recycled and retained within the plant by CAM (Madsen 1987) is appropriate to *C. helmsii*. A possible reason for the possession of CAM may be that the diffusive resistance to CO₂ inherent in the structure of the leaf of many of these types of plants, has allowed CAM to become established as a means of capitalising on readily available CO₂. Other aquatic CAM plants exhibit up to 98% re-assimilation of endogenous CO₂ released by the decarboxylation of malic acid (Madsen 1987), indicating a large capacity for capture and retention of CO₂ within the plant.

Associated with CAM is the possible benefit of enhanced N-use efficiency ($\text{mol C fixed mol}^{-1} \text{N s}^{-1}$) (Madsen 1987; Richardson et al. 1984). The elevation of CO₂ at the site of carboxylation, whether by adjacent decarboxylation of malic acid or by the prevention of loss of CO₂ from the plant, will inhibit photorespiration, and hence increase N-use efficiency by enhancing the efficiency of RUBISCO. The low rates of photosynthesis in all aquatic CAM plants indicate that there is a large resistance to CO₂-fixation (Black et al. 1981; Salvucci and Bowes 1982), which is the result of low RUBISCO activity in aquatic CAM plants (Farmer et al. 1986), and a high diffusion resistance to CO₂ uptake. Any alleviation of these restrictions by a reduction in investment in RUBISCO protein without a significant loss of carbon-assimilating capacity would be advantageous.

Enrichment of ¹³C in the growth medium failed to enhance the resolution of NMR spectra achieved using growth media of natural abundance ¹³C. This may indicate that the CO₂ fixed during the night prior to the measurement of the spectra did not come from the external medium, but from an internal source. We assume that more than 90% of the relevant -COOH of malic acid has come from CO₂ fixed in the night immediately before measurement. If CO₂ from respiration is recycled by re-

tention within the plant by fixation into malic acid or isocitric acid, then any additional external ^{13}C would contribute a relatively small amount to the total net C fixation. It may be possible to enhance the resolution of an NMR spectrum of ^{13}C within this plant by exposing the plant to ^{13}C for more than one dark period. This experiment may elucidate further the exact pathway of "fixed" inorganic C within the plant.

The assimilation of DIC during 75% of each 24-h period implies a resource acquisition advantage, which is directly linked to the possession of CAM. DIC-saturated photosynthetic rates of aerial leaves are similar to those of submerged leaves (Table 3), so access to atmospheric CO_2 , with reduced diffusion resistance (10^4 times less), does not increase the photosynthetic rate. CAM appears to maximise the potential for DIC acquisition in a diffusion-limited environment (water).

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