Marine Biology (2005) 147: 1085–1090
DOI 10.1007/s00227-005-0019-0

RESEARCH ARTICLE

Jacqueline Uku · Sven Beer · Mats Björk

Buffer sensitivity of photosynthetic carbon utilisation in eight tropical seagrasses

Received: 23 August 2004 / Accepted: 4 April 2005 / Published online: 13 July 2005

Abstract Some of the mechanisms involved in inorganic carbon (Ci) acquisition by tropical seagrasses from the western Indian Ocean were described by Björk et al. (Mar Biol 129:363–366, 1997). However, since then, it has been found that an additional, buffer-sensitive, system of Ci utilisation may operate in some temperate seagrasses (Hellblom et al. in Aquat Bot 69:55–62, 2001, Hellblom and Axelsson in Photos Res 77:173–191, 2003); this buffer sensitivity indicates a mechanism in which electrogenic H^+ extrusion may form acidic diffusion boundary layers, in which either HCO_3^-–H^+ is co-transported into the cells, or where HCO_3^- is converted to CO_2 (as catalysed by carbonic anhydrase) prior to uptake of the latter Ci form. Because a buffer was used in the 1997 study, we found it important to reinvestigate those same eight species, taking into account the direct effect of buffers on this potential mode of Ci acquisition in these plants. In doing so, it was found that all seagrass species investigated except *Cymodocea serrulata* were sensitive to 50 mM TRIS buffer of the same pH as the natural seawater in which they grew (pH 8.0). Especially sensitive were *Halophila ovalis*, *Halodule wrightii* and *Cymodocea rotundata*, which grow high up in the intertidal zone (only ca. 50–65% of the net photosynthetic activity remained after the buffer additions), followed by the submerged *Enhalus acoroides* and *Syringodium isoetifolium* (ca. 75% activity remaining), while *Thalassia hemprichii* and *Thalassodendron ciliatum*, which grow in-between the two zones, were less sensitive to buffer additions (ca. 80–85% activity remaining). In addition to buffer sensitivity, all species were also sensitive to acetazolamide (AZ, an inhibitor of extracellular carbonic anhydrase activity) such that ca. 45–80% (but 90% for *H. ovalis*) of the net photosynthetic activity remained after adding this inhibitor. Raising the pH to 8.8 (in the presence of AZ) drastically reduced net photosynthetic rates (0–14% remaining in all species); it is assumed that this reduction in rates was due to the decreased CO_2 concentration at the higher pH. These results indicate that part of the 1997 results for the same species were due to a buffer effect on net photosynthesis. Based on the present results, it is concluded that (1) photosynthetic Ci acquisition in six of the eight investigated species is based on carbonic anhydrase catalysed HCO_3^- to CO_2 conversions within an acidified diffusion boundary layer, (2) *C. serrulata* appears to support its photosynthesis by extracellular carbonic anhydrase catalysed CO_2 formation from HCO_3^- without the need for acidic zones, (3) *H. ovalis* features a system in which H^+ extrusion may be followed by HCO_3^-–H^+ co-transport into the cells, and (4) direct, non-H^+-mediated, uptake of HCO_3^- is improbable for any of the species.

Communicated by O. Kinne, Oldendorf/Luhe

J. Uku
Kenya Marine and Fisheries Research Institute, P.O. Box 81651, Mombasa, Kenya

M. Björk · J. Uku (✉)
Department of Botany, Stockholm University, 10691, Stockholm, Sweden
E-mail: uku@botan.su.se
Tel.: +46-8-163846
Fax: +46-8-165525

S. Beer
Department of Plant Sciences, Tel Aviv University, Tel Aviv, 69978, Israel

Introduction

The use of organic buffers is common in experimental work conducted to determine inorganic carbon (Ci) utilisation in seagrasses (reviewed in Beer et al. 2002). These buffers are used so as to maintain constant pH levels, which ensure a constant CO_2/HCO_3^- ratio within the experimental enclosures. Recent experiments on the temperate seagrasses *Zostera marina* (Hellblom et al. 2001) and *Ruppia cirrhosa* (Hellblom and Axelsson 2003) have, however, revealed the presence of a buffer sensitive mechanism for Ci utilisation, which is thought

to function through the electrogenic extrusion of H^+ across the plasma membrane (similarly to what was earlier described for *Chara corallina*, Lucas et al. 1983; Price and Badger 1985). This leads to localize acidification of the diffusion boundary layer (including the cell wall), followed either by HCO_3^-–H^+ co-transport (or HCO_3^-–OH^- antiport) into the cells, or carbonic anhydrase catalysed conversion of HCO_3^- to form CO_2, the latter of which then diffuses into to the cells (see Beer et al. 2002 for a description of the various systems of HCO_3^- utilisation in seagrasses). Thus, as buffers have been used in many of the past Ci utilisation experiments, the possible contribution of H^+ to Ci utilisation in the plants investigated may have been missed.

Björk et al. (1997) studied eight tropical seagrass species with the aim of determining their Ci utilisation mechanisms. In that work, the carbonic anhydrase inhibitor acetazolamide (AZ) was used in order to detect if extracellular activity of the enzyme was required as part of a Ci utilisation system in which HCO_3^- was dehydrated to CO_2 prior to uptake of the latter Ci form. However, both the controls and AZ treated samples were measured in the presence of a buffer so as to keep pH and, thus, the HCO_3^-/CO_2 ratio constant during the experiments. Based on the above-mentioned revelations that photosynthetic Ci acquisition of temperate seagrasses could depend on H^+ extrusion, it is apparent that the use of buffers may have masked a similar process also in those plants. Therefore, we undertook the present study so as to re-evaluate the effect of buffer on the Ci utilisation mechanism(s) in the same tropical seagrasses that were used in that earlier work by Björk et al. (1997). In doing so, it was found that indeed buffers might inhibit H^+ extrusion and, thus, photosynthetic Ci acquisition in many tropical seagrasses.

Materials and methods

This study was undertaken in Zanzibar, Tanzania (western Indian Ocean, 06°10′S, 39°20′E). The seagrasses used were collected during low tide mornings from Mbweni Beach, located ca. 5 km south of Zanzibar Town. The species used were *Halophila ovalis* (R. Br.) Hook. f., *Halodule wrightii* Ascherson, *Cymodocea rotundata* Ehrenberg and Hempr.ex Ascherson, *C. serrulata* (R. Br.) Ascherson and Magnus, *Thalassia hemprichii* (Ehrenberg) Ascherson, *Thalassodendron ciliatum* (Forskål) den Hartog, *Enhalus acoroides* (L.) Royle and *Syringodium isoetifolium* (Ascherson) Dandy. A few shoots of each species were collected daily from the field, and kept in seawater under dim light conditions until the experiments were run later the same day. 1 M stock solutions of tris (hydroxymethyl) aminomethane (TRIS) were prepared and adjusted to either pH 8.0 or pH 8.9. These buffers yielded pH 8.0 and 8.8, respectively, when mixed with seawater to a final concentration of 50 mM. A stock solution of 20 mM AZ was prepared by dissolving the powder in 50 mM NaOH. Three Hansatech (UK) DW1 O_2 electrode chambers were used for determining net rates of O_2 evolution (here termed net photosynthesis). The chambers were illuminated using fibre optical light guides (Schott, Germany) such that the plant material in each chamber received a saturating irradiance of 1,200 μmol photons m^{-2} s^{-1}. The chambers were filled with 2.5 ml natural seawater (pH 8.0), and the solutions were stirred vigorously with a magnetic follower throughout the experiments. For each seagrass species, one epiphyte free leaf was cut into 20-mm sections for insertion into the chambers, with the exception of *H. ovalis* and *H. wrightii*. In the case of *H. ovalis*, two whole leaves were used while for *H. wrightii* up to ten leaves were combined together in the chambers. The leaf sections were inserted into the chambers in a bent "u-shape" such that they were maximally exposed to the light.

In the first set of experiments, the leaves were enclosed in the chambers, and net photosynthetic rates were allowed to stabilise (within 5–10 min). A small amount of TRIS buffer (pH 8.0, so as to form a final concentration of 50 mM) was then injected into the electrode chambers, and the net photosynthetic rates were again left to stabilise so as to assess the buffer effect on possible H^+ extrusion. Thereafter, AZ was added to a final concentration of 0.1 mM to measure the combined effect of the buffer and of inhibiting extracellular activity of carbonic anhydrase. In a second set of experiments, using fresh plant material, 0.1 mM (final concentration) AZ was added to the seawater after the initial rate of photosynthesis in natural seawater had been determined, so as to measure the effect of inhibiting extracellular carbonic anhydrase only. In both sets of experiments, the pH was maintained at 8.0 during the ca. 15-min duration of the measurements whether or not the buffer was used. In the third set of experiments, after the initial seawater run with fresh plant material, the high pH TRIS buffer (pH 8.9) was added to a final concentration of 50 mM (resulting in a final pH of 8.8) together with AZ. This was done in order to reduce the equilibrium CO_2 concentration to ca. 20% that of normal seawater while still keeping the HCO_3^- concentration high (Schwarz et al. 2000). While the addition of AZ likely affected the hydration rate of CO_2 to become spontaneously slow (since extracellular carbonic anhydrase activity was inhibited), the fact that photosynthetic rates of all seagrasses were drastically reduced within minutes of increasing the pH indicates that it was fast enough to reduce the diffusion of CO_2 into the photosynthesising cells. Under the latter conditions, it was also assumed that HCO_3^- could not be dehydrated at any considerable rates and that HCO_3^-–H^+ co-transport could not occur (as extruded H^+ would be neutralised by the buffer) and, thus, that the only form of Ci utilisation could be via non-H^+-mediated HCO_3^- transport.

After the experiments, the lengths and widths of the incubated plant parts were measured in order to base

photosynthetic rates on leaf area. The net photosynthetic responses to TRIS or/and AZ were expressed as percentages of the net photosynthetic rate in natural seawater. The percentage net photosynthetic responses of each seagrass were transformed using the arcsine transformation, after which *t* tests were performed to determine if the addition of TRIS and/or AZ had a significant effect on the net photosynthesis of each seagrass in natural seawater. The significance level was set at $P < 0.05$.

Results

The effects of TRIS buffer and/or AZ at pH 8.0, and the combined effects of TRIS buffer and AZ at pH 8.8, on the photosynthetic rates of the different seagrasses are shown in Fig. 1. All species except *C. serrulata* showed significant responses to the buffer additions. The sensitivity to buffer was especially marked for *H. ovalis*, *H. wrightii* and *C. rotundata* (62, 50 and 67% net photosynthetic O_2 evolution, respectively, remaining after the buffer addition). The other species were less sensitive to the buffer addition: *S. isoetifolium* and *E. acoroides* showed a remaining 76% net photosynthetic activity, and *T. hemprichii* and *T. ciliatum* 84 and 81%, respectively. Addition of AZ, to the buffer-inhibited leaves caused a further significant decline in photosynthetic rates. In the experiment where only AZ was added, this inhibitor caused a marked decline in photosynthetic rates for all species except *H. ovalis*, where, although significant, the effect was low. These results indicate that both H^+ extrusion and catalysis via extracellular carbonic anhydrase stimulate, to various degrees, Ci acquisition in most species. The effects of buffer and AZ were usually additive such that the rates resulting from the addition of both inhibitors were, in most cases, close to those calculated from the addition of each one separately. This was not the case for *H. ovalis*, where the addition of AZ had a strong effect only after the addition of TRIS (but not alone).

Tris (hydroxymethyl) aminomethane buffer together with AZ at the high pH value of 8.8 drastically reduced the net photosynthetic rates of all plants (Fig. 1). At this pH, only ca. 20% of the original seawater CO_2 concentration is maintained in equilibrium with HCO_3^-, and the possible contribution of such a low CO_2 concentration would be further impeded as the activity of carbonic anhydrase (and, thus, the possible re-supply rate of CO_2 close to the plasma membrane was inhibited, as was in our contention the H^+ extrusion system by the presence of the buffer). Thus, the most feasible mode of Ci utilisation at such conditions would be direct, H^+ independent (non-electrogenic), HCO_3^- uptake. However, less than 15% photosynthesis remained under those conditions, excluding the possibility that such apparent HCO_3^- transport significantly contributed to photosynthesis in these seagrasses.

Discussion

In the initial study of Ci utilisation in the same tropical seagrass species as investigated here, Björk et al. (1997) used an organic buffer (HEPES) in order to maintain the normal Ci concentration ratio at a stable level during the experiments. The results of that study showed that the photosynthetic responses of all species except *S. isoetifolium* were inhibited by AZ, and *H. wrightii, C. rotundata, C. serrulata* and *E. acoroides* were especially inhibited. While this indicated the importance of extracellular carbonic anhydrase catalysed HCO_3^- to CO_2 conversion prior to uptake of the latter Ci form, it is apparent that the additional effect of H^+ extrusion had been missed because of the use of a buffer. In our present study, where the buffer effect was separated from the effect of AZ, it seems that the formation of acidic zones together with the activity of carbonic anhydrase are both important components of Ci acquisition in most of these tropical seagrasses. Thus, *H. wrightii*, *C. rotundata*, *T. hemprichii*, *T. ciliatum*, *S. isoetifolium* and *E. acoroides* seem to depend on carbonic anhydrase catalysed conversions of HCO_3^- to CO_2 within the acidic zones formed by H^+ extrusion. This would place them in HCO_3^- utilisation system "c" as defined by Beer et al. (2002, see Fig. 2), i.e., a system in which carbonic anhydrase catalyses the conversions of HCO_3^- to CO_2 within the acidified diffusion boundary layer prior to uptake of the latter Ci form. The advantage of such HCO_3^- to CO_2 formation within acidic zones is that the Ci equilibrium CO_2 concentration is higher there than at normal seawater pH, thus favouring its inward diffusion. In *C. serrulata* (where no buffer effect, but only a significant AZ effect, was detected), it seems that HCO_3^- to CO_2 conversions as catalysed by extracellular activity of carbonic anhydrase in non-acidified zones is enough to support the photosynthetic rates measured. This would place this plant in HCO_3^- utilisation system "a" (Fig. 2). In *H. ovalis*, the effect of AZ was low, as was also reported by Schwarz et al. (2000) on the basis of in situ measurements of photosynthetic electron transport. It thus seemed possible that electrogenic H^+ extrusion only could drive photosynthesis in this species in accordance with system "b" (Fig. 2). In this system, it is envisioned that the extruded protons are used for subsequent co-transport of HCO_3^- into the photosynthesising cells. On the other hand, the strong effect of AZ after TRIS addition shows that carbonic anhydrase is involved in the Ci uptake system of this plant too, possibly indicating the presence of two parallel systems (a + b, Fig. 2). In most cases, the effects of buffer and AZ were additive within a limit of 13%, i.e. the percentage inhibition with both inhibitors together could be approximately derived by adding their respective effects. However, in two cases, i.e. for *H. ovalis* and *C. rotundata*, the measured effect of both inhibitors together was much stronger than that calculated based on each inhibitor separately. While we can offer no clear expla-

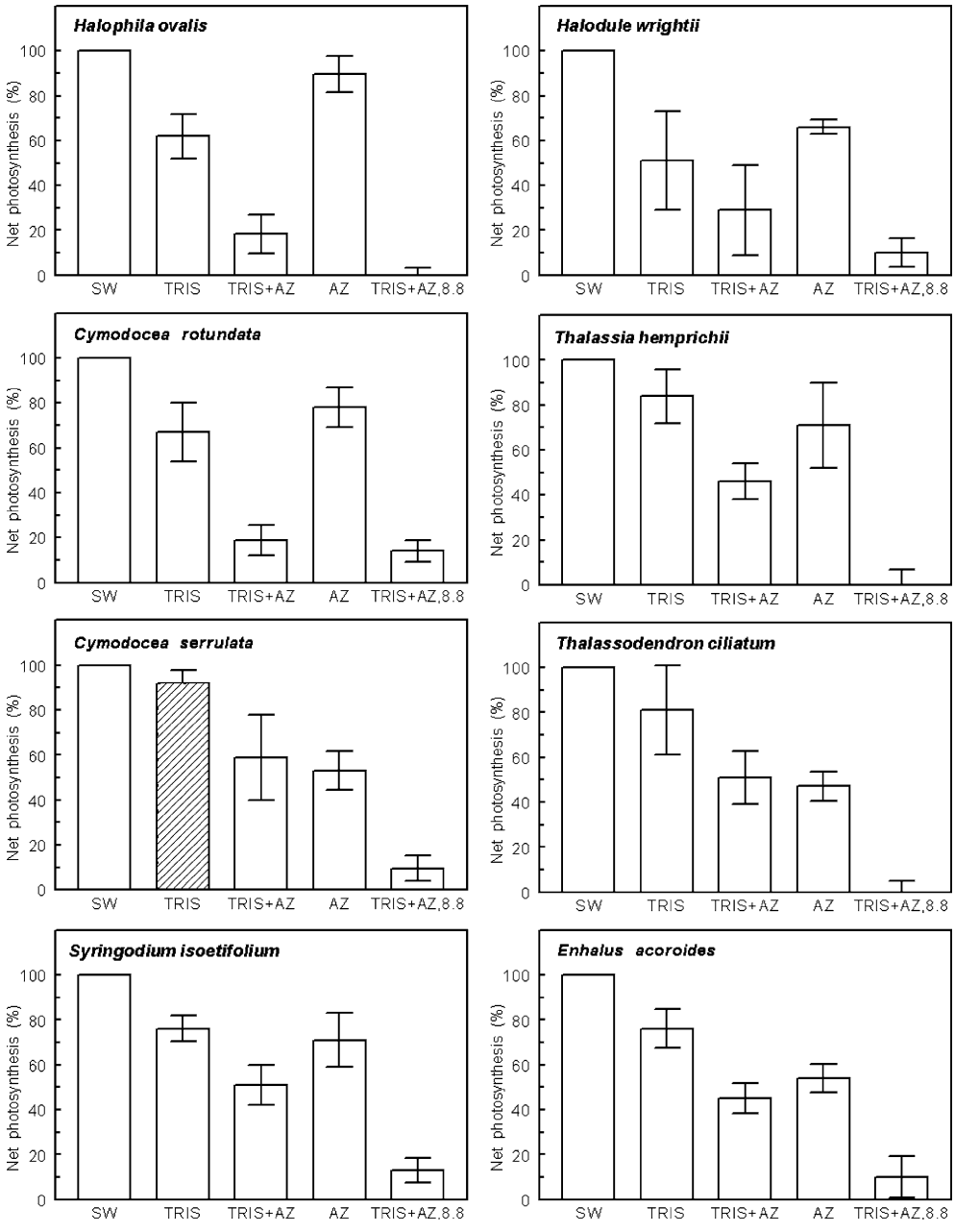

Fig. 1 Net photosynthetic responses of the eight seagrass species, relative to control rates in normal seawater (100%, represented by the *SW bar*), when subjected to 50 mM TRIS buffer at pH 8.0 (TRIS, *second bar from the left*), followed by acetazolamide (TRIS + AZ, *third bar*), AZ only (*fourth bar*) as compared to a new control (also represented by the *SW bar*) and TRIS buffer with AZ at pH 8.8 (TRIS + AZ,8.8, *fifth bar*) as compared to a new control (also represented by the *SW bar*). Data are the average of six replicates (two runs with three electrode chambers) ± SD. All open TRIS bars are significantly different from the SW bars, all TRIS + AZ bars are significantly different from both the SW and the TRIS bars and all AZ bars are significantly different from the SW bars, as are all SW + TRIS, 8.8 bars (*t* test, $P < 0.05$). The *striped TRIS bar* for *Cymodocea serrulata* indicates no significant difference from the SW bar. The average (± SD) net photosynthetic rates in normal seawater (representing 100% in the SW bars, in µmol O_2 m^{-2} s^{-1}) were for *Halophila ovalis* 2.0 (± 0.14), *Halodule wrightii* 0.9 (± 0.12), *C. rotundata* 2.0 (± 0.41), *Thalassia hemprichii* 1.0 (± 0.23), *C. serrulata* 0.9 (± 0.09), *T. ciliatum* 0.7 (± 0.05), *Syringodium isoetifolium* 1.0 (± 0.11), and for *E. acoroides* 0.7 (± 0.10)

nation for this, it may be that carbonic anhydrase mediated HCO_3^- dehydration was less important than creating the H^+ gradient (especially for *H. ovalis*, see above), or that there are synergistic effects of one system on the other, the nature of which, however, remains unknown.

The experimental approach to this work relies largely on the effects of TRIS buffer and AZ, and on increasing the pH to 8.8. While inhibitory effects of TRIS buffer per se on intracellular membrane transport systems have been reported (Gordon-Weeks et al. 1997; Dobrovinskaya et al. 1999), it is our contention that the effect here was extracellular. This is indicated by the fact that one seagrass species (*C. serrulata*) was not significantly affected by the buffer, and more drastic effects would have been expected also for the other species were TRIS to enter the cells. Also, it was found in initial tests here that *H. ovalis* showed the same sensitivity to HEPES as to TRIS buffer (results not shown). Similarly to TRIS, also AZ acts extracellularly (Maren 1984; Moroney et al. 1985), thus inhibiting the activity of carbonic anhydrase bound to the outer part of the plasma membrane.

It was previously reported that some seagrasses could transport HCO_3^- into their photosynthesizing cells. For *Z. marina*, in addition to a large effect of AZ, dicyclohexyl-carbodiimide inhibited photosynthetic rates by 35%, indicating ATPase dependent HCO_3^- transport across the plasma membranes (Beer and Rehnberg 1997). However, again, an organic buffer was used in combination with that inhibitor, questioning today that

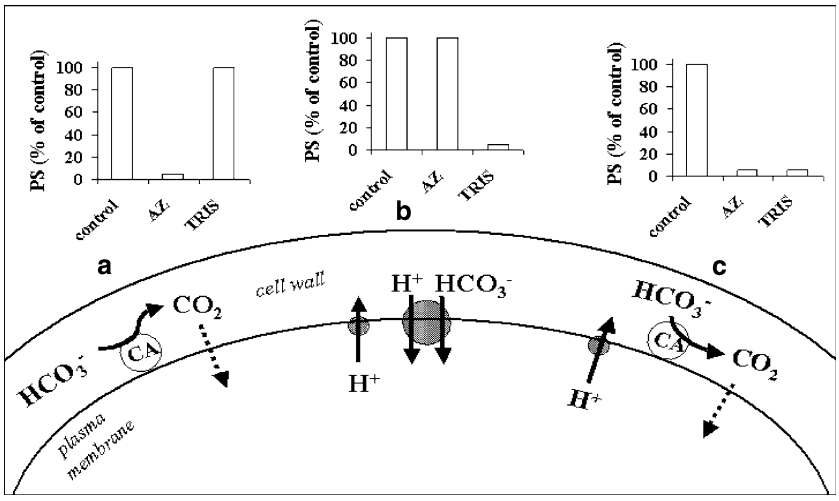

Fig. 2 Illustration of three different possible systems for HCO_3^- utilisation in seagrasses as indicated by their photosynthetic responses to the extracellular inhibitor of carbonic anhydrase, AZ and a H^+ neutralising buffer (TRIS). Taken from Beer et al. (2002)

such a HCO_3^- uptake system was in effect (as based on the subsequent finding of buffer sensitive photosynthesis in this plant, Hellblom et al. 2001). Similarly, the possibility of HCO_3^- transport was reported for *H. ovalis*, *C. rotundata* and *S. isoetifolium* as based on their ability to photosynthesise at pH 8.6 above what would be expected at the ca. 30% CO_2 remaining in equilibrium with HCO_3^- at that pH as compared to normal seawater (Björk et al. 1997). In the present work, however, it was unlikely that direct, non-H^+-mediated, HCO_3^- transport was in effect. This is indicated by the findings that photosynthetic rates in all species were inhibited to well below the 20% that could have been accounted for by the minimum CO_2 concentration potentially remaining in equilibrium with HCO_3^- at that high pH.

It is interesting to note that photosynthetic rates of the species growing high up in the intertidal (*H. ovalis*, *H. wrightii* and *C. rotundata*) were most strongly inhibited by buffer and, thus, seemed to depend particularly on H^+ extrusion and the formation of acidic zones within their diffusion boundary layers. These plants are exposed to high irradiances during the day, and H^+ extrusion may confer to them an efficient means for Ci acquisition under such conditions. In the case of *H. ovalis*, it is possible that the proposed mode of Ci utilisation (according to system b, supported by a, Fig. 2), confer particularly efficient means of saturating its photosynthetic system with CO_2. This is further indicated by the findings that photosynthetic electron transport of this seagrass is Ci saturated by the normal seawater Ci concentration (Schwarz et al. 2000), and that it maintains high rates of electron transport even during extremely high midday irradiances without showing significant signs of photoinhibition (Beer and Björk 2000). For the other species, including the subtidal ones (except *C. serrulata*), the proposed acidified zones within the diffusion boundary layer formed by H^+ extrusion may be beneficial for carbonic anhydrase mediated HCO_3^- to CO_2 conversions within those zones since, at the low pH, CO_2 would be formed at much higher Ci equilibrium concentrations than if acidification did not occur. Thus, while in earlier works it was unclear how high enough CO_2 concentrations could be established by the action of only extracellular activity of carbonic anhydrase at close(r) to normal seawater pH, it now seems to be clear that carbonic anhydrase usually acts in a lower-pH environment, thus creating high enough CO_2 concentrations to account for high rates of photosynthesis. If this system is present in some temperate seagrasses (Hellblom et al. 2001; Hellblom and Axelsson 2002), then its importance for maintaining high photosynthetic rates in tropical seagrasses, growing in environments where high temperatures and high irradiances are conducive to high rates of productivity, is even more apparent.

Acknowledgements We thank Mia Bengtson for excellent assistance in both field collections and laboratory work. We also thank the personnel of IMS, Zanzibar, for their assistance and for the use of their facilities. Financial support was provided by Sida/SAREC.

References

Beer S, Björk M (2000) A comparison of photosynthetic rates measured by pulse amplitude modulated (PAM) fluorometry and O_2 evolution in two tropical seagrasses. Aquat Bot 66:69–76

Beer S, Rehnberg J (1997) The acquisition of inorganic carbon by the seagrass *Zostera marina*. Aquat Bot 56:277–283

Beer S, Björk M, Hellblom F, Axelsson L (2002) Inorganic carbon utilization in marine angiosperms (seagrasses). Funct Plant Biol 29: 349–354

Björk M, Weil A, Semesi S, Beer S (1997) Photosynthetic utilization of inorganic carbon by seagrasses from Zanzibar, East Africa. Mar Biol 129:363–366

Dobrovinskaya OR, Muniz J, Pottosin II (1999) Asymmetric block of the plant vacuolar Ca^{2+}-permeable channel by organic cations. Eur Biophys J 28:552–563

Gordon-Weeks R, Korenkov VD, Steele SH, Leigh RA (1997) Tris is a competitive inhibitor of $K^=$ activation of the vacuolar H^+-pumping pyrophosphatase. Plant Physiol 114:901–905

Hellblom F, Axelsson L (2003) External HCO_3^- dehydration maintained by acid zones in the plasma membrane is an important component of the photosynthetic carbon uptake in *Ruppia cirrhosa*. Photos Res 77:173–191

Hellblom F, Beer S, Björk M, Axelsson L (2001) A buffer sensitive inorganic carbon utilization system in *Zostera marina*. Aquat Bot 69:55–62

Lucas WJ, Keifer DW, Sanders D (1983) Bicarbonate transport in *Chara corallina*: Evidence for cotransport of HCO_3^- with H^+. J Membr Biol 73:263–274

Maren TH (1984) The general physiology of reactions catalyzed by carbonic anhydrase and their inhibition by sulphonamides. Ann N Y Acad Sci 429:568–579

Moroney JV, Husic HD, Tolbert NE (1985) Effects of carbonic anhydrase inhibitors on inorganic carbon accumulation by *Chlamydomonas reinhardtii*. Plant Physiol 79:177–183

Price GD, Badger MR (1985) Photosynthetic utilization of bicarbonate in *Chara corallina*. Aus J Plant Physiol 12:257–267

Schwarz AM, Björk M, Buluda T, Mtolera M, Beer S (2000) Photosynthetic utilization of carbon and light by two tropical seagrass species as measured in situ. Mar Biol 137:755–761