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Elodea canadensis under N and CO_2 Limitation: Adaptive Changes in Rubisco and PEPCase Activity in a Bicarbonate User

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S u m m a r y

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Diffusion of CO_2 in water is 10,000 times slower than in air. Because of this photosynthesis in submerged aquatic macrophytes is often limited by CO_2 availability. *Elodea canadensis* shows HCO_3^- utilization under conditions of CO_2 limitation. A closely related species, *Hydrilla verticillata*, which also belongs to the Hydrocharitaceae, shows a C_4 like mechanism when grown under so-called summer conditions where low availability of CO_2 is expected. Here it is shown that a similar C_4-like mechanism is possibly also present in *E. canadensis*. To study the possible interference between the two processes, *E. canadensis* was grown at low and high CO_2 and NO_3^-, conditions expected to affect HCO_3^- utilization. Moreover the effect was studied of these growth conditions on the occurrence of the C_4 state. A low G_{CO2} was observed in *E. canadensis* when it was grown under low CO_2 conditions indicative for plants in the C_4-state. The low G_{CO2} was related to a change in the ratio Rubisco and PEPCarboxylase indicating a central role of these enzymes. The very same conditions induced HCO_3^- utilization in this species. Both mechanisms thus operate in the same plant simultaneously. Growing *E. canadensis* at high NO_3^-, 0.75 mM, had a strong stimulatory effect on O_2 production in the light both in low and high CO_2 grown plants, suggesting that it has no direct relation with HCO_3^- utilization. The actual cause for this stimulatory effect of high N on photosynthesis is unclear.

I n t r o d u c t i o n

Submerged aquatic macrophytes (SAM) are often faced with limitation of their photosynthesis by low availability of inorganic carbon, e.g. dissolved CO_2.

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Diffusion of CO_2 in water is about 10,000 times slower than in air. Furthermore, the major component of the dissolved inorganic carbon (DIC) pool is HCO_3^- at pH 7-9, common for natural waters. Since only CO_2 diffuses freely over the plasmamembrane to the cytoplasm, other inorganic carbon species have to be converted into CO_2 or need an active transport system across the plasmamembrane to contribute to carbon fixation. To describe the inorganic carbon system, the following terms are used: CO_2, dissolved free CO_2 including H_2CO_3; HCO_3^-, bicarbonate; CO_3^{2-}, carbonate; DIC: sum of CO_2, H_2CO_3, HCO_3^- and CO_3^{2-}.

The slow diffusion of gases in water also generates a high O_2 level in the photosynthesizing cells of submerged aquatic plants. The resulting low CO_2/O_2 ratio near the Rubisco site is favorable to photorespiration and accordingly would lead to a low rate of carbon fixation. In the course of evolution several SAM responded to this challenge by development of mechanisms to use HCO_3^- as an alternative carbon source and/or mechanisms to concentrate inorganic carbon (for review see BOWES & SALVUCCI 1989, PRINS & ELZENGA 1989). Mechanisms from the first category are associated with an increased potential for HCO_3^- utilization either by active uptake of HCO_3^- in the leaf cells or by conversion of HCO_3^- into CO_2. The second category are CO_2-concentrating mechanisms (CCM), based on a C_4- or CAM-like metabolism. Often these mechanisms of enriching the cell with CO_2 are accompanied by an increased carbonic anhydrase (CA) activity in the apoplast of leaf cells.

Models for HCO_3^- utilization in fresh water macrophytes are generally based on active H^+ extrusion into the apoplast driven by a plasmalemma bound ATPase. Two mechanisms have been proposed. The first described mechanism is a light dependent acidification of the apoplast and unstirred layer near the leaf surface (PRINS & al. 1982). This acidification results in a shift of the equilibrium between HCO_3^- and CO_2 in the direction of CO_2 which then diffuses into the leaf cells. To balance the loss of H^+ there is an H^+ influx or OH^- efflux at another part of the leaf. There is no agreement if the alkaline zones result from H^+ influx or OH^- efflux or by another process. For convenience the expression H^+ influx is used here.

This mechanism has now been observed in a number of fresh water angiosperms. E.g., in *Elodea*, *Egeria*, and *Potamogeton* there generally is an acidification at the abaxial side of the leaf to pH values of 4-5 and an alkalization at the adaxial side to pH values or 9-11 (PRINS & ELZENGA 1989). A second mechanism has been proposed for a.o. the Characeae. The basis for this latter mechanism is HCO_3^- transport by symport with H^+, driven by the proton motive force and accompanied by acid regions of H^+ efflux and alkaline regions of H^+ influx (LUCAS 1985).

A C_4-like metabolism has been proposed for *Hydrilla verticillata* and other SAM. Typical C_4-characteristics are induced in *Hydrilla* when grown under conditions normally expected to lead to high rates of photorespiration. SAM showing this C_4-like metabolism are, like their terrestrial counterparts, characterized by a low CO_2-compensation point (Γ_{CO2}), a photosynthesis relatively insensitive to O_2 and a low Rubisco/PEPCase (phosphoenolpyruvate) activity ratio (R/P), due to a high PEPCase activity (SALVUCCI & BOWES 1981, HOLADAY & al. 1983, BOWES &

SALVUCCI 1989). In this species, Rubisco is localized in the chloroplast and PEP-Case in the cytoplasm (REISKIND & al. 1989). After fixation of inorganic carbon by PEPCase in the cytoplasm, the concentrating of DIC occurs most likely in the chloroplast (REISKIND & al. 1997).

In *Elodea canadensis* acidification is induced by conditions of high light and low DIC in plants previously grown under conditions favorable to photorespiration. Acidification is absent in *Elodea* plants grown under high CO_2 conditions (ELZENGA & PRINS 1989). This observation led to the question: is acidification in *Elodea* accompanied by C_4-characteristics, such as a low Γ_{CO2} and a low R/P, as observed in *Hydrilla*. A low Γ_{CO2} could facilitate the diffusion of CO_2 into the leaf cells, following the conversion of HCO_3^- into CO_2 by the process of acidification (PRINS & al. 1982).

It is generally thought that the main physiological effect of HCO_3^- utilization is an increase of the rate of carbon fixation. It may also contribute to suppression of photorespiration through an increased availability of CO_2 and thereby, to a more efficient use of nitrogen (BEARDALL & al. 1982, RAVEN 1985, RAVEN & LUCAS 1985). To answer the above question, experiments were set up to study the effect of low and high NO_3^-, in combination with low and high CO_2 during the growth period, on acidification, Γ_{CO2} and R/P. It was hypothesized that high CO_2 and high NO_3^- during growth would lead to a relatively high Γ_{CO2} and a high R/P and the adverse conditions to low values for Γ_{CO2} and R/P. The combination of low and high CO_2 and low and high NO_3^- might also give insight in the mechanism, which induces acidification when CO_2 levels are low during growth. Under conditions of high NO_3^-, low CO_2 will limit the rate of carbon fixation and thereby growth. Under conditions of low NO_3^-, growth may be limited by N availability rather than by the rate of carbon fixation.

Materials and Methods

Elodea canadensis Michx. was grown in tanks on a clay substrate covered with sand (VAN GINKEL & PRINS 1998). For the present experiments sprouts of around 100 mm were transferred to 2-liter Erlenmeyer flasks containing an appropriate culture solution. Each flask contained about 15 sprouts, which were cultured for 1 to 3 weeks in 5% Hoagland growth medium. This solution contained 0.75 mM NO_3^- (further referred to as high N) or 0.15 mM, one fifth of this concentration, (referred to as low N) at low or high CO_2. Low CO_2 was obtained by continuous aeration with normal air and high CO_2 by constant titration of the growth medium to pH 6.6 by bubbling with CO_2 (ELZENGA & PRINS 1989). The medium was changed every fourth day. The temperature was 21 °C (± 1 °C) and the fluence rate 60 µmol m^{-2} s^{-1} PAR with a photoperiod of 12 hours.

Rubisco and PEPCase were assayed in a crude extract made from newly formed shoots. The extraction buffer contained 60 mM HEPES, 24 mM $MgCl_2$, 30 mM $KHCO_3$, 0.24 mM NaEDTA and 6 mM DTT. The pH was set to pH 8.0 by KOH for Rubisco and to pH 7.1 for PEP-Case (adapted from REISKIND & BOWES 1991). The shoots were first frozen in liquid N_2 and thereafter ground in the presence of 5% (w/w) Polyclar-AT$^{(TM)}$. The ground plant material was suspended in extraction buffer of pH 7.1 or 8.0. The ratio plant material/extraction buffer was 1:10 (w/v). Three aliquots of 50 µl were taken and added to 950 µl 85% acetone for chlorophyll determination (LICHTENTHALER 1987). Thereafter the plant debris was spinned down in an Eppendorf centrifuge (14,000 rpm, 10 min.). Aliquots of 100 µl supernatant were tested for enzyme activity. The

assay for both enzymes was spectrophotometrically and based on a decrease in NADH concentration, measured as absorption at 340 nm. The Rubisco assay was as described by BESFORD 1983. The PEPCase assay was based on a method described by STITT & al. 1989. The buffer used for this assay contained 50 mM HEPES, 20 mM $MgCl_2$, 25 mM $KHCO_3$, 0.2 mM NaEDTA, 5 mM DTT and was brought at pH 8.0 with KOH. The reaction was started by adding phosphoenolpyruvate (PEP, final concentration 8 mM) after a preincubation at 30 °C. The enzyme activity was determined after 10 minutes incubation at 30 °C. The Γ_{CO2} (at 21 % O_2) was determined with an infra red gas analyzer (HOLADAY & al. 1983). The experimental solution was a 5% Hoagland medium buffered with 50 mM MES brought at pH 5.5 with KOH. This pH was chosen so that CO_2 was the only carbon source and to prevent any contribution by HCO_3^- (VAN & al. 1976). During the experiment the pH remained constant, the fluence rate was 300 µmol m^{-2} s^{-1} PAR and the temperature 21 °C. The light induced pH-polar reaction was measured in artificial pond water, APW, containing 5 mM $CaCl_2$, 1.5 mM KCl, 1 mM NaCl and $KHCO_3$ as indicated (PRINS & al. 1982).

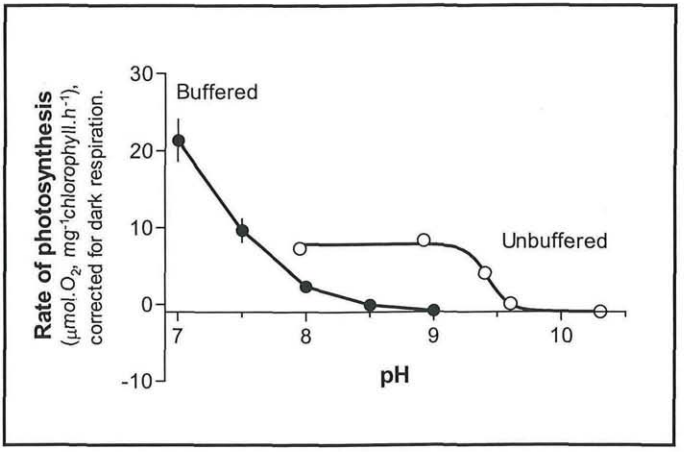

Fig. 1. Light induced O_2 production rate, corrected for dark respiration, of *E. canadensis* leaves plotted against pH using either a strongly buffered (25 mM MES/BTP) or an unbuffered APW solution containing 1 mM HCO_3^-.

Photosynthetic O_2 production at different pH's was measured using an O_2 electrode. The experimental solution, consisting of APW and 25 mM MES/BTP buffer, was rapidly stirred. The combination of stirring and strong buffering should effectively prevent any use of HCO_3^- as carbon source, as the build up of an acid region by polarity was prevented. To test this, net O_2 production was measured in a strongly buffered and an unbuffered experimental solution of APW. With buffering, O_2 production stopped completely at pH 8.5, while in APW it continued till pH 9.5 (Fig. 1).

Results

The water in the tanks, wherein the plants originally were grown before the low and high CO_2 culture period, was weakly aerated by normal air and has to be considered 'low CO_2'. Accordingly the plant material directly taken from the tanks showed pH-polarity, with abaxial acidification under CO_2 limiting conditions. Transferred to the Hoagland growth medium the *Elodea* sprouts grew vigorously,

especially in the high CO_2 solutions, and new sprouts (and roots) developed. The pH of the low CO_2 growth medium increased to values of around 10 in the light and returned in the dark to around 7, as shown in the inset of Fig. 2 for *Elodea* grown at low N. This drift of the pH, while the solution was aerated with normal air, indicated a high capacity of *Elodea* for extracting DIC from its surrounding medium (ADAMEC 1993, SAND-JENSEN & GORDON 1986). In accordance herewith *Elodea*, grown in 5% Hoagland under low CO_2 showed the typical pH polar reaction, when transferred to APW + 1 mM $KHCO_3$ pH 8, light 60 µmol m^{-2} s^{-1}, (Fig. 2) as described earlier (ELZENGA & PRINS 1989). When *Elodea* was grown under high instead of low CO_2 conditions no pH polarity was induced and there was only a small a-polar increase of the pH near both leaf sides and the change to adaxial alkalization and abaxial acidification did not occur (Fig. 2). At this stage no difference between low and high N plants was observed.

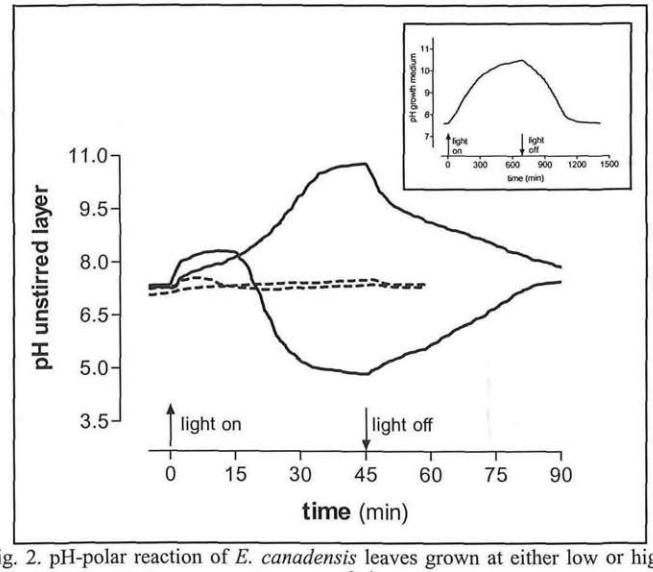

Fig. 2. pH-polar reaction of *E. canadensis* leaves grown at either low or high CO_2. Medium: 5% Hoagland low N, fluence rate 60 µmol $m^{-2}s^{-1}$ PAR. Inset: Culture solution pH of low CO_2 culture, one light-dark cycle (24 hours).

A shift to a C_4-like state was observed in *Elodea* when the plants were cultured under low CO_2 conditions. The Γ_{CO2} was low for the low CO_2 plants compared to the Γ_{CO2} of plants cultured at high CO_2. In the low CO_2 plants R/P shifted from Rubisco to PEPCase (Table 1). Fig. 3 shows the actual Rubisco and PEPCase activity for plants after being transferred to the Hoagland culture solutions of low and high N and C for 1 to 2 weeks. The results for high and low N are described below.

Table. 1. Γ_{CO2} in ($\mu l\ l^{-1}$ ± SEM), chlorophyll content in mg g FW^{-1} ± SEM, c_a/c_b and R/P of *E. canadensis* grown in 5% Hoagland, normal or 1% NO_3^- (high or low N) for 2-3 weeks, under high and low CO_2 availability. *) The Γ_{CO2} for low CO_2 plants at either high or low N was not significantly different.

	High CO_2 High N	Low CO_2 High N	High CO_2 Low N	Low CO_2 Low N
Chlorophyll	2.53 ± 0.34	3.27 ± 0.36	2.31 ± 0.16	2.61 ± 0.18
Ca/Cb	2.77 ± 0.02	2.69 ± 0.05	2.79 ± 0.03	2.68 ± 0.02
Γ_{CO2}	70-90	20-30*)	70-90	30-40*)
R/P	2.09 ± 0.35	1.25 ±0.41	3.10 ± 0.33	1.88 ± 0.17

The Rubisco activity remained constant in the low CO_2, but increased in the high CO_2 plants. The PEPCase activity in contrast, remained nearly constant in the high as well as in the low CO_2 plants (Fig. 3). As a result the R/P was relatively high in these high N plants grown at high CO_2, but low in those grown at low CO_2 (Fig. 3; Table 1). This corresponded with a high Γ_{CO2} in the high CO_2 plants and a low Γ_{CO2} in the low CO_2 plants (Table 1).

The Rubisco activity increased in both low and high CO_2 grown plants (Fig. 3). The PEPCase activity increased in the low CO_2 plants, while it showed only a slight, probably not significant, downward tendency in the high CO_2 plants (Fig. 3). As a result the activity ratio of Rubisco/PEPCase (R/P) of the low N plants showed the same tendency as in high N plants, R/P was high in high CO_2 grown plants and low in those grown at low CO_2 (Fig. 3). As with the high N plants this corresponded with a high Γ_{CO2} in the high CO_2 plants and a low Γ_{CO2} in those grown at low CO_2 (Table 1).

Net O_2 production by Elodea was measured, using a strongly buffered experimental solution with a constant DIC concentration of 1 mM and a varying pH. Under these conditions photosynthesis depends entirely dependent on the CO_2 with no contribution of HCO_3^- (Fig. 4). Under these conditions no difference was ob served between the low and high CO_2 plants, of either low or high N grown plants. Therefore the low and high CO_2 data were pooled. In contrast there was a very marked difference between the low and high N plants. The rate of O_2 production of the high N plants at the highest external [CO_2] was twice that of the low N plants. In the low N plants photosynthetic O_2 production was saturated between 0.1 and 0.2 mM CO_2 while in the high N plants saturation was not yet reached at 0.5 mM CO_2.

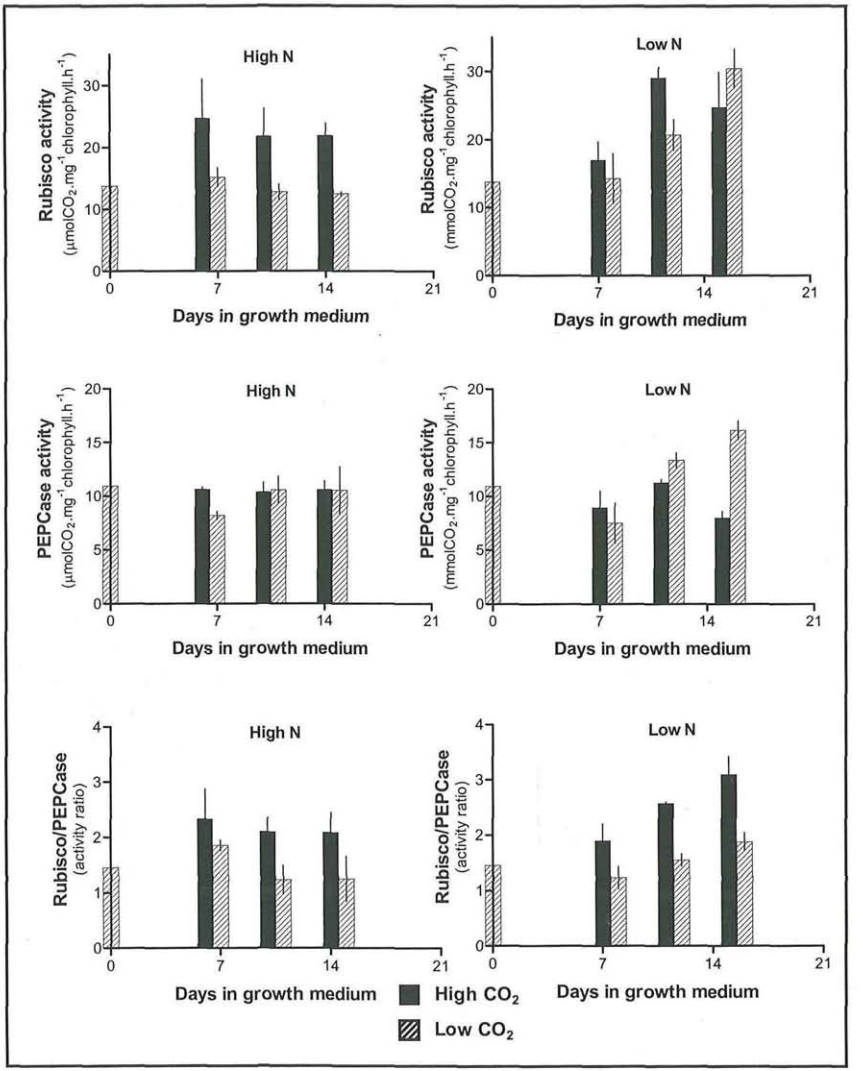

Fig. 3. Rubisco and PEPCase activity of *E. canadensis* shoots grown in 5% Hoagland solution for 1-2 weeks, at high or low N and low or high CO_2, fluence rate 60 μmol m^{-2} s^{-1} PAR, photoperiod 12 h, 21 °C. 1-2 weeks.

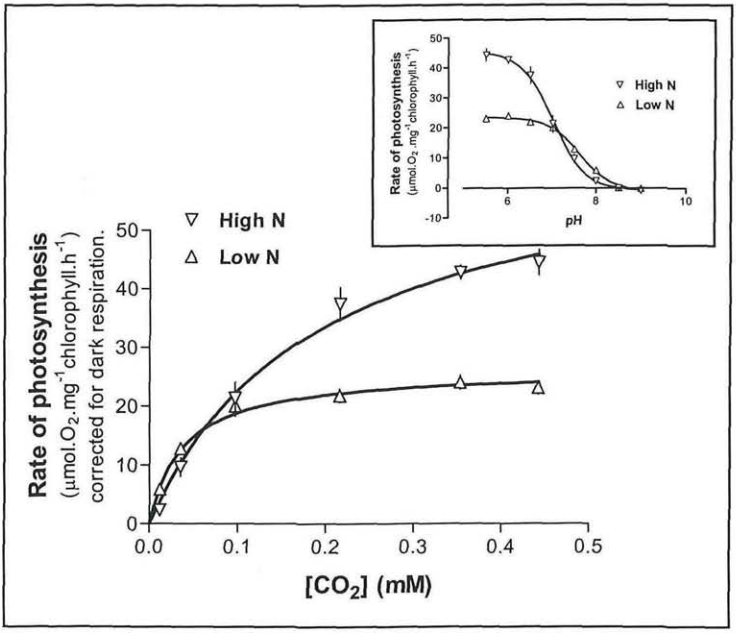

Fig. 4. Light induced O_2 evolution of *E. canadensis* leaves at 60 µmol m^{-2} s^{-1} versus $[CO_2]$, [DIC] was constant, 1 mM. $[CO_2]$ was varied by changing the pH, see *inset*.

The chlorophyll content and the chlorophyll a/b ratio, c_a/c_b, depended on the growth conditions (N, CO_2) of the plants. High CO_2 plants grown at either low or high N, did not significantly differ in their chlorophyll content. When plants were grown at low CO_2 high N plants contained far more chlorophyll than low N plants, and also more than the plants grown at high CO_2, either at low or high N. A higher c_a/c_b was observed in plants grown at high CO_2 compared to plants grown at low CO_2 (Table 1). The different growth conditions resulted in plants with similar dry/fresh weight ratios of around 8%. Furthermore both, low and high CO_2 plants (leaves) had a similar protein content. The low N plants, both low and high CO_2, had a lower protein content, 3-4 µg protein g FW^{-1}, than high N plants, 7-8 µg protein g FW^{-1}.

Discussion

Light induced pH polarity is suppressed in *Elodea* grown at high CO_2 in both low (Fig. 2) and high N cultured plants (data not shown, but see ELZENGA & PRINS 1989), in agreement with the inhibition of bicarbonate utilization (ADEMEC 1993). At 0.4 mM external $[CO_2]$ the rate of O_2 production of high N grown plants was twice that of low N plants. No difference was observed between low and high

CO_2 plants. Strong pH buffering makes acidification by pH-polarity ineffective and therefore, CO_2 was the only C source in these experiments (PRINS & al. 1982). The stimulation of O_2 production by high N growth conditions did not result from an increased chlorophyll content as the rate of O_2 production is expressed on a chlorophyll basis.

High CO_2 growth conditions induced a high Γ_{CO2}, in both high and low N plants (JAHNKE & al. 1991, ADAMEC 1993). The compensation point of low CO_2 grown *Elodea* seemed somewhat higher in high than in low N grown plants. This may be related to a higher rate of net O_2 production observed in high N grown plants and a twice as high protein content of these leaves.

The data on Rubisco and PEPCase activity show that R/P was higher in the high than in low CO_2 plants. The underlying changes in Rubisco and PEPCase activity differ between low and high N *Elodea* plants. The growth condition in the tanks, from which the original starting plant material was collected, was low CO_2. Accordingly the Rubisco activity increased under high CO_2 in high N plants conditions while it remained the same in low CO_2 plants. The PEPCase activity of the high N plants did change however, neither in the low CO_2 nor in the high CO_2 plants. As a result R/P increased when plants were grown under high N, high CO_2 conditions. In the low N plants the changes are less clear although they lead to a similar change in R/P: Rubisco activity increased under both low and high CO_2 conditions; PEPCase activity of low N plants only increased in the low CO_2 grown plants. No significant change was observed in high CO_2 grown plants. The combined effect is an increase of R/P in the low N, high CO_2 grown plants. An observed increase of both Rubisco and PEPcase activity under low CO_2 conditions in low N plants indicates a more specific effect of low N during growth.

E. canadensis is taxonomically closely related to *H. verticillata*, both species belong to the Hydrocharitaceae family. The species are morphologically very similar, both are fresh water submerged macrophytes and both show photosynthetic HCO_3^- utilization and pH polarity (ELZENGA & PRINS 1986,1989, REISKIND & al. 1997). In *Hydrilla* C_4-like characteristics were induced by so-called summer growth conditions: high light and temperature and low CO_2 (HOLADAY & al. 1983, BOWES & SALVUCCI 1989). Typical for C_4 like *Hydrilla* are a low Γ_{CO2} and a low R/P activity ratio due to a relatively high PEPCase activity compared to C_3 like *Hydrilla.* In *Elodea* pH polarity and the C_4 characteristics of a low Γ_{CO2} and a relatively high R/P ratio were all induced by growing the plants under low CO_2 conditions, while fluence rate and temperature were kept constant. This effect of high CO_2 growth conditions may seem comparable to some extent to the effect of winter and summer conditions on *Hydrilla*. One of the characteristics of winter conditions, which lead to C_3 plants characterized by a high Γ_{CO2}, is a higher availability of CO_2 compared to summer conditions which lead to C_4 plants with a low Γ_{CO2}, (SALVUCCI & BOWES 1981, HOLADAY & al. 1983, BOWES & SALVUCCI 1989). However, winter growth conditions did not suppress pH-polarity in *Hydrilla*. The observation of local acidification, leading to the conversion of HCO_3^- into CO_2, and C_4-like characteristics induced in both species by low CO_2, raises the question how these two processes cooperate or interact. Given the similarities between both spe-

cies it is assumed that the mechanism of CCM in *Hydrilla* is also active in *Elodea*. Activity of such a C_4-like CCM could result in a lowering of cytoplasmic CO_2 producing a steeper gradient for CO_2 between apoplast and cells leading to a higher photosynthetic rate. Although this seems an attractive hypothesis it is not entirely supported by the experiments on photosynthetic O_2 production. The O_2 production as determined in these experiments reflected CO_2 fixation without HCO_3^- utilization. Under conditions of an active CCM therefore, one would expect a higher rate of CO_2 fixation and thus a higher rate of O_2 production by low CO_2 plants. This was not observed, neither in the low N nor in the high N plants. Assuming that CCM also occurred in *Elodea* it did not result in a higher rate of O_2 production, in contrast to the use of HCO_3^- via acidification, which leads to an increased O_2 production (PRINS & al. 1982). Clearly the two mechanisms, CCM and pH-polarity, contribute in different ways to photosynthesis in SAM like *Elodea* and *Hydrilla*. CCM activity seems to result in suppression of photorespiration but has only a marginal effect, if any, on the net rate of photosynthesis when diffusion of CO_2 into the leaf is limiting.

The cause of the stimulatory effect of high N during growth on photosynthetic O_2 production in both low and high CO_2 plants remains unclear. Possibly low N during growth in low CO_2, but not in high CO_2, induces a C_4-like state, i.e. it lowers the Γ_{CO2} and the R/P ratio, which may indicate that suppression of photorespiration is related to a more efficient use of N (RAVEN 1985). MADSEN & BAATTRUP-PEDERSEN 1995 showed that in *Elodea* Rubisco activity was directly related to tissue-N level, and activity was substantially higher than could be expected on the basis of photosynthetic capacity. Furthermore, the relation between initial slope of the CO_2 response curve and Rubisco activity was linear. So clearly Rubisco had a regulatory effect under CO_2-limiting circumstances and a similar conclusion can be drawn from the present results.

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