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Regular paper: Biochemical and biophysical CO_2 concentrating mechanisms in two species of freshwater macrophyte within the genus *Ottelia* (Hydrocharitaceae)

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Key words: Bicarbonate use, CAM, C_4 metabolism, organic acids, photosynthesis

Abbreviations: AMP: Adenosine monophosphate; ATP: Adenosine triphosphate; Alk: alkalinity; CAM: Crassulacean Acid Metabolism; CCM: carbon dioxide concentrating mechanism; DIC: dissolved inorganic carbon; DTT: dithiothreitol; FW: fresh weight; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; LDH: lactate dehydrogenase; MDH: malate dehydrogenase; NAD(P)-ME: NAD(P)-malic enzyme; OAA: oxaloacetate; PEP: phosphoenol pyruvate; PEPC: PEP carboxylase; PEPCK: PEP carboxykinase; PGK: phosphoglycerate kinase; PPDK: pyruvate phosphate dikinase; RuBisCO: ribulose 1,5-bisphosphate carboxylase-oxygenase; RuBP: ribulose 1,5-bisphosphate.

Abstract

Two freshwater macrophytes, *Ottelia alismoides* and *Ottelia acuminata,* were grown at low (mean 5 µmol L^{-1}) and high (mean 400 µmol L^{-1}) CO_2 concentrations under natural conditions. The ratio of PEPC to RuBisCO activity was 1.8 in *O. acuminata* in both treatments. In *O. alismoides*, this ratio was 2.8 and 5.9 when grown at high and low CO_2, respectively, as a result of a 2-fold increase in PEPC activity. The activity of PPDK was similar to, and changed with, PEPC (1.9-fold change). The activity of the decarboxylating NADP-malic enzyme (ME) was very low in both species while NAD-ME activity was high and increased with PEPC activity in *O. alismoides*. These results suggest that *O. alismoides* might perform a type of C_4 metabolism with NAD-ME decarboxylation, despite lacking Kranz anatomy. The C_4-activity was still present at high CO_2 suggesting that it could be constitutive. *O. alismoides* at low CO_2 showed diel acidity variation of up to 34 µequiv g^{-1} FW indicating that it may also operate a form of Crassulacean Acid Metabolism (CAM). pH-drift experiments showed that both species were able to use bicarbonate. In *O. acuminata*, the kinetics of carbon uptake were altered by CO_2 growth conditions, unlike in *O. alismoides*. Thus the two species appear to regulate their carbon concentrating mechanisms differently in response to changing CO_2. *O. alismoide*s is potentially using three different concentrating mechanisms. The Hydrocharitaceae have many species with evidence for C_4, CAM, or some other metabolism involving organic acids, and are worthy of further study.

Introduction

All eukaryotic photoautotrophs, plus their cyanobacterial predecessor, assimilate CO_2, via the Calvin Benson-Bassham or reductive pentose phosphate cycle where the carboxylation reaction is catalyzed by ribulose 1,5-bisphosphate carboxylase-oxygenase (RuBisCO; EC 4.1.1.39; (Raven et al. 2012)). However, RuBisCO, has a relatively low affinity for CO_2 and will also fix oxygen competitively leading to subsequent further carbon loss via photorespiration (Bowes et al. 1971; Ogren 2003). Some terrestrial plants have a biochemical carbon dioxide concentrating mechanism (CCM), whereby carbon is fixed by the oxygen insensitive phosphoenol pyruvate carboxylase (PEPC; EC 4.1.1.31) producing a C_4 acid, oxaloacetate (OAA), that is rapidly converted into malate or aspartate. The C_4 acid is then decarboxylated, to produce elevated concentrations of CO_2 around RuBisCO, minimising photorespiration (Hatch and Slack 1966 ; Raghavendra and Sage 2011). Most terrestrial C_4 plants have 'Kranz anatomy', where the initial carboxylation by PEPC is spatially separated from subsequent decarboxylation and RuBisCO fixation, in order to prevent futile cycling (Raghavendra and Sage 2011). However, a dramatic variant of C_4 plant was discovered in a submersed monocot, *Hydrilla verticillata* (Hydrocharitaceae; Holaday and Bowes 1980; Bowes 2011) that operates an inducible single-celled C_4 metabolism with CO_2 concentrating in the chloroplast. About a decade ago, C_4 metabolism was also described in single-cells of two land plants (Chenopodiaceae), *Biernertia cycloptera* and *Borszczowia aralocapsica* with RuBisCO and PEPC in different parts of the same cell (Edwards et al. 2004; Voznesenskaya et al. 2002; Voznesenskaya et al. 2001).

Three major sub-types of C_4 plants have been described based on the decarboxylation step that liberates CO_2 from the C_4 acid compounds. In two sub-types, malate is decarboxylated to form CO_2 and pyruvate, one with NADP-malic enzyme (NADP-ME, EC 1.1.1.40) and one with NAD-malic enzyme (NAD-ME, EC 1.1.1.39). The pyuvate re-enters the cycle via pyruvate phosphate dikinase (PPDK EC 2.7.9.1) that regenerates phosphoenolpyruvate, the substrate for PEPC. In a third sub-type, PEP carboxykinase (PEPCK, EC 4.1.1.49) decarboxylates OAA to form CO_2 and PEP. In

the plants with NAD-ME or PEPc kinase, aspartate rather than malate is shuttled from the mesophyll to the bundle sheath cells (Raghavendra and Sage 2011).

Other terrestrial plants, especially those associated with arid environments, possess Crassulacean Acid Metabolism (CAM) where there is a temporal separation of carbon-fixation by PEPC and RuBisCO. In these plants, PEPC is active at night causing malate to accumulate within the vacuole. This C_4 acid is then decarboxylated during the day to produce CO_2 that is fixed by RuBisCO. This is primarily a water conserving mechanism minimising gaseous exchange during the day but it also serves to conserve carbon by reducing respiratory carbon loss (Cushman and Bohnert 1999; Silvera et al. 2010).

Concentrations of CO_2 in lakes frequently exceed air-equilibrium as a result of input from the catchment of CO_2 or terrestrially fixed organic carbon that is oxidised to CO_2 (Cole et al. 2007; Maberly et al. 2013). However, in productive systems the rate of carbon-fixation in a unit volume of water can greatly exceed rates of carbon supply from the atmosphere, or other sources, leading to depletion of CO_2 virtually to zero (Maberly 1996) limiting productivity (Ibelings and Maberly 1998; Jansson et al. 2012). Furthermore, the rate of CO_2 diffusion in water is about 10^4-times lower in water than in air (Raven 1970) leading to substantial transport limitation through the boundary layer surrounding objects in water (Black et al. 1981). As a consequence, the concentration of CO_2 needed to half-saturate the net photosynthesis of freshwater macrophytes is roughly 8 to 14 times air-equilibrium (Maberly and Madsen 1998).

Freshwater macrophytes have a range of avoidance, amelioration or exploitation strategies to overcome the problem of limited inorganic carbon supply (Klavsen et al. 2011).The most frequent CCM in freshwater macrophytes is based on the biophysical use of bicarbonate (Maberly and Madsen 2002). Bicarbonate is the most abundant form of inorganic carbon in all freshwaters where the pH is between about 6.3 and 10.1: the first and second dissociation constants of the carbonate system. Even when concentrations of CO_2 are strongly depleted as a result of photosynthetic carbon uptake, concentrations of bicarbonate can still be substantial. Freshwater concentrations of bicarbonate range

from zero in acid systems to over 100 mmol L^{-1} in soda lakes (Talling 1985). The use of bicarbonate, like other CCMs, is an active process requiring the expenditure of energy and may involve 'polar leaves' with localised areas of proton extrusion leading to conversion of bicarbonate to CO_2 and subsequent inward diffusion, or direct uptake of bicarbonate (Elzenga and Prins 1987).

Although much less widespread, some freshwater macrophytes also possess a type of C_4 metabolism. The best studied is that of the dioecious form of the hydrocharitacean *Hydrilla verticillata* (Bowes 2011; Holaday and Bowes 1980) that operates an inducible single-celled C_4 mechanism based on carbon fixation by PEPC and decarboxylation by NADP-ME, in addition to being able to use bicarbonate. Similar, albeit less well characterised, C_4 mechanisms appear to operate in other monocotyledons: *Egeria densa* (Hydrocharitaceae; (Browse et al. 1977; Casati et al. 2000), and in amphibious species *Eleocharis vivipara* (Cyperaceae) (Ueno et al. 1988), and *Orcuttia viscidia*, *Neostapfia colusana* and *Tuctoria greenii* (Poaceae; Keeley and Sandquist 1992)).

A number of freshwater macrophytes have also been shown to possess CAM (Keeley 1981, 1998). These include species within the genus *Isoetes* (Lycopodiophyta), and the angiosperms *Littorella uniflora* (Madsen 1987b, a), *Crassula helmsii* (Newman and Raven 1995) (Klavsen and Maberly 2009) and *Vallisneria spiralis* (Keeley 1998). Underwater, CAM acts as a carbon-conserving mechanism that reduces the loss of respiratory carbon at night and exploits the nocturnal concentrations of CO_2 that are often higher than during the day (Klavsen et al. 2011).

In terrestrial plants, the global frequency of C_4 is about 3% (Edwards et al. 2004) and that of CAM about 6%, (Silvera et al. 2010) with the remainder (91%) being C_3 and so lacking CCMs. In contrast, about 55% of aquatic angiosperms have a biophysical CCM based on HCO_3^- use and others have a biochemical CCM based on CAM (4%) or C_4 (3% ; Maberly and Madsen 2002).

The Hydrocharitaceae contains a number of species with biochemical CCMs, including the C_4 syndrome (e.g *Hydrilla verticillata*, *Egeria densa*) or CAM activity (e.g *Vallisneria spiralis*) but many ecologically important species within this family have not been studied. One species-rich genus within the Hydrocharitaceae that has been little studied is *Ottelia* Pers. Here we characterized the

CCMs of two species from China, *Ottelia acuminata,* (Gagne.) Dandy var. lunanensis H. Li and *Ottelia alismoides* (Linn.) Pers., and tested their ability to acclimate to different concentrations of CO_2.

Material and Methods

Plant material and growth conditions

Ottelia acuminata and *O. alismoides* were both collected from Yunnan Province, China and then cultivated in a greenhouse in Wuhan Botanical Garden for several years. Seeds were germinated in a growth chamber (temperature 25°C) and when the seedlings reached about 20 cm tall, they were transferred to 10 cm diameter plant pots containing sediment from nearby Donghu Lake and placed inside glass tanks (30 x 40 x 60 cm tall) containing about 65 litres of tap water with an alkalinity of about 2 mequiv L^{-1}. The glass tanks were located in a glasshouse on the flat roof of the laboratory in larger tanks (about 400 L) of running water to reduce diurnal changes in water temperature. The experiment was started on 11 July 2012 and finished on 29 September 2012. During the experimental period, snails and moribund leaves were removed every day. Two or three times each day, water temperature was recorded and a water sample collected to measure pH with a combination pH electrode (Metrohm 6.0238.000, Herisau, Switzerland) connected to a meter (Metrohm 718 STAT Titrino).

Two treatments were produced with four replicate tanks per treatment, each containing both species. In the 'low CO_2' treatment, the natural photosynthetic activity of the plants was allowed to deplete the inorganic carbon concentration of the water and increase the pH. In the 'high CO_2' treatment, tank water saturated with CO_2 was added to the tanks two to three times each day to reduce the pH to between 6.6 and 7.0 and thereby increase the concentration of CO_2. The tanks were gently stirred to mix the water after each addition of CO_2 solution. Both treatments were out of equilibrium with air CO_2 and although CO_2 concentrations varied over time, the concentrations in the two treatments were very different.

Enzyme activity measurements

Leaves were harvested, blotted dry and quickly weighed to determine fresh weight, and then frozen in a pestle and mortar with liquid nitrogen. Typically, about 0.6 g fresh weight (FW) of leaf was extracted and 3 mL of ice-cold extraction buffer was added for each gram FW of leaf. The extraction buffer comprised 50 mM Tris, 0.1 mM EDTA, 15 mM $MgCl_2$, pH 8 (buffer A) plus 10% glycerol. Following grinding to a smooth paste, the whole extract was centrifuged at 5°C for 45 minutes at 12,000 g (Heraeus, Biofuge Fresco, Germany). The supernatant (the crude extract) was stored on ice prior to measuring enzyme activity.

RuBisCO activity was measured in crude extracts by coupling its activity to NADH oxidation using phosphoglycerate kinase from yeast (PGK; Sigma St Louis, MO USA) and glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle (GAPDH; Sigma). Prior to measuring activity, the extract was incubated in buffer A in the presence of 20 mM bicarbonate for 5 min. Activity was then followed using buffer A with 0.2 mM NADH (Sigma), 1 mM ATP (Sigma), 5 mM DTT (Shanghai Chemical Reagents Company, China), 5 units of PGK and 5 units of GAPDH and 1 mM ribulose 1,5-bisphosphate (Sigma). The disappearance of NADH was followed at 340 nm using a UV-Vis spectrophotometer (TU-1810PC, Purkinje General, China). The calculated carboxylase activity took account of the fact that two molecules of NADH are oxidized for every molecule of RuBP catalyzed.

PEPC activity was measured using buffer A, with 20 mM bicarbonate and 1 mM phosphoenol pyruvate (Sigma) to produce oxaloacetate that is in turn coupled to malate dehydrogenase (MDH, Sigma) activity using an excess of this enzyme. The reaction mixture therefore also contained 0.2 mM NADH and 5 units of MDH. Activity was continuously followed by recording a decrease of absorbance at 340 nm.

NAD-ME activity was measured spectrophotometrically in buffer A supplemented with 1 mM NAD (Biosharp, Japan), 10 mM malate (Energy Chemical, Shanghai, China), 1 mM $MnCl_2$, and 5 mM dithiothreitol. NADP-ME activity in crude extracts was measured spectrophotometrically in

buffer A containing 1.5 mM NADP (Sigma), 10 mM malate; 1 mM $MnCl_2$, and 5 mM dithiothreitol. Activities of NAD-ME and NADP-ME were followed continuously by recording an increase of absorbance at 340 nm.

PPDK activity was measured spectrophotometrically, at 340 nm, in the opposite direction to the one operating in C_4 photosynthesis, by following pyruvate formation and NADH disappearance using lactate dehydrogenase (LDH, Amresco, Biochemicals and Life Science Research Products). The reaction was carried out in buffer A supplemented with 5 mM PEP, 1.2 mM AMP (Sigma), 1 mM pyrophosphate, 2.5 mM dithiothreitol, 0.2 mM NADH and 2 units of LDH.

All activities were maximal activities for the studied growth conditions but are not *in vivo* activities.

CAM activity

The daily change in titratable acidity was calculated as the difference between the minimum and maximum amount of titratable acidity per unit fresh mass. The minimum amount of acid was measured on plants collected at the end of the pH-drift experiment (July) or collected towards the end of the light period on 14-16 August and 27-29 September 2012. The maximum amount of acid was assayed after incubation of material in the dark at 25°C for 18 hours in 1 mmol L^{-1} equimolar $NaHCO_3$ and $KHCO_3$ at a concentration of CO_2 of about 700 µmol L^{-1} (pH about 6.4). About 0.2 g fresh mass of material was quickly blotted, carefully weighed, roughly chopped into 10 mL plastic stoppered tubes and frozen at -20 °C. Prior to analysis, 5 mL of deionised water was added and the tubes were boiled for 15 minutes, cooled and stored in a refrigerator. Titratable acidity was assayed on an aliquot from each tube by end-point titration to pH 8.3 using approximately 0.01 mol L^{-1} NaOH, standardized by Gran titration against 0.1 mol L^{-1} HCl. Measurements were made in triplicate and results are expressed as µequiv g^{-1} FW.

pH-drift

The ability of leaves to use bicarbonate was assessed in pH-drift experiments (Maberly and Spence 1983). Leaves were cleaned by gentle rubbing to remove the marl deposit from their upper surface. They were then rinsed for about 10 minutes in one of the two test media: equimolar concentration of $NaHCO_3$ and $KHCO_3$ at total HCO_3^- concentrations of 0.1 or 1.0 mmol L^{-1}. The leaves were placed in 30 mL test tubes with ground glass stoppers containing 25 mL of solution and about 5 mL air. The tubes were incubated in a growth cabinet at a constant temperature of 25°C and receiving about 75 μmol photon m^{-2} s^{-1} (photosynthetically available radiation) from fluorescent tubes, measured with a cosine corrected sensor (Li-Cor LI-192SA). The pH was measured after 24 hours and roughly every 6 to 12 hours thereafter until a maximum pH was reached. The final alkalinity in the solution was measured by Gran titration with a standard solution of HCl.

Kinetics of O_2 evolution

Rates of net photosynthesis were measured as O_2 evolution at 25°C at a photon irradiance of 120 μmol photon m^{-2} s^{-1}. Leaves (0.2 to 0.5 g fresh weight) were collected from the growth tanks, and cleaned by gentle rubbing to remove the marl deposit from their upper surface. They were then rinsed for about 10 minutes in a solution of 20 mM Tricine, pH 7. The leaves were then placed in a glass and Perspex chamber, the volume of which was 120 mL, in Tricine buffer bubbled briefly with nitrogen (starting O_2 concentration 60 to 70% air saturation). The chamber was sealed and the O_2 concentration measured with an optical oxygen electrode (YSI Pro ODO Yellow Spring Instruments, USA) calibrated in air at 100% humidity and 25°C. Incremental small volumes (6 to 90 μL) of 2 mol L^{-1} $Na/KHCO_3$ stock were added to generate a range of inorganic carbon concentrations from 0.1 to 3.8 mmol L^{-1}. The output of the electrode was logged on a computer and linear regressions of concentration against time were used to calculate rates of oxygen exchange. The kinetic response was fitted to the Michaelis-Menten equation.

Soluble protein, chlorophyll and leaf area

The soluble protein concentration of crude extracts was assayed using the Bio-Rad (Hercules, CA, USA) reagent using bovine serum albumin as a standard (Bradford 1976). The content of chlorophyll *a* and *b* in the leaves of *Ottelia* was determined on 0.1 to 0.5 g fresh leaf material (n = 3 to 6). Chlorophyll was extracted overnight at 4°C with 95 % ethanol and chlorophyll concentration was calculated from absorbance measured in a spectrophotometer (TU-1810PC) using the equations of (Brain and Solomon 2007). Projected (1 –sided) leaf area was calculated from digital photographs using AreaAna software (Huazhong University of Sciences and Technology, China).

Results

Growth conditions

The temperature was identical (around 29°C) in the low and high CO_2 treatments and relatively constant (Table 1). The pH in the low CO_2 treatment was more than one pH unit greater than in the high CO_2 treatment. Precipitation of calcium carbonate on the leaves of both species of *Ottelia* in the low CO_2 treatment caused the alkalinity to be on average nearly one mequiv L^{-1} lower than in the high CO_2 treatment. The bicarbonate concentration in the low CO_2 treatment was consequently also lower than that in the high CO_2 treatment. The CO_2 concentration was 80-fold lower in the low *vs* the high CO_2 treatment.

Soluble protein, chlorophyll and leaf area

Growth in low or high CO_2 did not have a statistically significant effect on the soluble protein, chlorophyll and leaf area of *O. alismoides*, although the ratio of chlorophyll *a* to chlorophyll *b* was slightly higher at high vs low CO_2 ($P<0.05$; Table 2). For *O. acuminata*, the chlorophyll content per unit fresh weight was 1.7-fold higher in leaves grown at low CO_2 compared to leaves grown at high CO_2 ($P<0.01$; Table 2).

Enzyme activities

The activity of RuBisCO on a protein basis was similar in both species and did not vary with the CO_2 growth conditions (Fig. 1A). In *O. alismoides,* PEPC activity was 2-fold higher in low CO_2 compared to high CO_2 leaves (Student's *t*-test, P<0.001) but was constant in *O. acuminata* (Fig. 1B). Consequently the ratio of PEPC to RuBisCO activity increased significantly from 2.8 to 5.9 in *O. alismoides* (Student's *t*-test, P<0.001) while it remained constant at about 1.8 in *O. acuminata* (Fig. 1C).

Pyruvate phosphate dikinase (PPDK), a key enzyme in two of the three decarboxylation types of C_4, showed a similar pattern of change to PEPC (Fig. 1D). The CO_2 concentration during growth did not affect PPDK activity in *O. acuminata* but triggered a 1.9-fold increase in *O. alismoides* at low compared to high CO_2 that was highly significant (Student's *t*-test, P<0.001). There was a significant correlation between activity of PEPC and PPDK (Fig. 2A). The activity of the widespread decarboxylating enzyme NADP-ME was very low in both species. The activity of NADP-ME at low and high CO_2 concentration during growth did not change in *O. alismoides*, but decreased at low CO_2 in *O. acuminata* (Student's *t*-test, P<0.05; Fig. 1E). Activity of NADP-ME did not correlate with changes in activity of PEPC (Fig. 2B). In contrast to NADP-ME, activities of NAD-ME (Fig. 1F) were very high and up to 27-fold greater than the activity of PEPC. In *O. acuminata* NAD-ME activity was slightly, but significantly, greater in the low CO_2-grown compared to high CO_2-grown leaves (Student's *t*-test, P<0.05). The pattern in *O. alismoides* was similar but difference between high and low CO_2 treatments were not significant (Student's *t*-test, P= 0.08). The activity of NAD-ME increased with that of PEPC in *O. alismoides* (Fig. 2C). We were not able to measure PEPCK spectrophotometrically as malate dehydrogenase was present in the crude extract and would interfere with the assay.

CAM capacity

The CAM capacity of the two species was assessed initially by measuring diel change in acidity. Across the two species and growth conditions for CO_2, acidity levels varied between 14 and 24 μequiv g^{-1} FW in the light and between 22 and 44 μequiv g^{-1} FW in the dark (Fig. 3). There was a statistically significant difference between light and dark acidity levels in *O. alismoides* at low CO_2 of about 24 μequiv g^{-1} FW (Student's *t*-test, $P<0.05$; Fig. 3). *O. alismoides* at high CO_2 showed a small diel change in acidity that was not statistically significant, but there was no evidence for diel acidity variation in *O. acuminata* in either condition.

In *O. alismoides*, the capacity to undertake CAM was re-measured in August and September. In August, a similar pattern was obtained and in leaves grown at low CO_2 there was a statistically significant (Student's *t*-test, $P<0.01$) diel change in acidity of 34 μequiv g^{-1} FW (Fig. 3b), slightly greater than in July. However, in September there was no indication of a diel acidity change in either CO_2 treatment (Fig. 3c). Even in the absence of a diel change in acidity, there was a substantial amount of acidity on all measuring occasions, 26 to 44 μequiv g^{-1} FW, at the end of the dark period.

pH-drift

The pH-drift experiments provided clear evidence for bicarbonate use in both species. The final concentration of bicarbonate was relatively constant and low with values between 0.06 and 0.09 mmol L^{-1} in the low CO_2 treatment and 0.06 and 0.11 mmol L^{-1} in the high CO_2 treatment (Table 3). The final CO_2 concentration was very low, in the range of 3 to 26 nmol L^{-1} and tended to be lower when measured at the higher concentration of bicarbonate which is again consistent with use of bicarbonate and lower than would be expected from C_4 photosynthesis alone based on CO_2 uptake. For example, assuming a low C_4 CO_2 compensation point of 3 ppm in air, at 25°C this would be equivalent to 100 nmol L^{-1}, roughly 4- to 30- times higher than the final CO_2 concentrations in the drift experiments.

There were small differences in the final CO_2 concentration between CO_2 treatments at the low bicarbonate test concentration, especially in *O. acuminata,* with lower final CO_2 concentrations in the low CO_2 treatment. There were substantial but reproducible shifts in alkalinity despite rinsing the

leaves several times in the test medium prior to the experiment. In the lower alkalinity experiment, alkalinity increased but in the higher alkalinity experiments, alkalinity was unchanged in the presence of *O. acuminata* but reduced in the presence of *O. alismoides.*

Kinetics of O_2 evolution

Oxygen exchange was measured as a function of dissolved inorganic carbon (DIC) concentration at pH 7 in both species and both treatments (Fig. 4). In *O. alismoides*, the kinetic responses of leaves from the low and high CO_2 treatments were not significantly different (variance ratio test; $F_{2,12}$ = 1.82, p = 0.20). Using the combined data, the maximum net rate of O_2 evolution was 27.2 (SD = 1.2) µmol O_2 g^{-1} FW h^{-1} which is equivalent to 56 and 39 µmol O_2 mg^{-1} Chla h^{-1} at high and low CO_2 respectively. The $K_{½}$ for DIC was 1.29 (SD = 0.15) mmol L^{-1} which at pH 7 is equivalent to 0.199 mmol L^{-1} CO_2 and 1.090 mmol L^{-1} HCO_3^-. In *O. acuminata*, the kinetic responses of leaves from the two treatments were significantly different (variance ratio test; $F_{2,12}$ = 5.00, $P < 0.05$). At the low CO_2 treatment, the maximum rate of O_2 evolution was 44.0 (SD = 3.9) µmol O_2 g^{-1} FW h^{-1} (48 µmol O_2 mg^{-1} Chla h^{-1}) and the $K_{½}$ for DIC was 1.64 (SD = 0.34) mmol L^{-1}. At the high CO_2 growth treatment, the maximum rate of O_2 evolution was 37.8 (SD = 4.9) µmol O_2 g^{-1} FW h^{-1} (69 µmol O_2 mg^{-1} Chla h^{-1}) and the $K_{½}$ for DIC was 2.36 (SD = 0.62) mmol L^{-1}. At pH 7, the $K_{½}$ at low and high CO_2 growth treatments are equivalent to 0.253 and 0.363 mmol L^{-1} respectively for CO_2 and 1.386 and 1.994 mmol L^{-1} respectively for HCO_3^-. The maximum rate of O_2 evolution was greater in *O. acuminata* than in *O. alismoides*, but the values of $K_{½}$ were between 1.27- and 1.82-fold greater in *O. acuminata* than in *O. alismoides*. If the maximum rate of O_2 evolution is expressed on a protein basis, however, the rates in the two species are very similar.

Discussion

Comparison of carbon concentrating mechanisms in *Ottelia* with other aquatic and terrestrial plants

Ottelia acuminata and *O. alismoides* both have the carboxylating, PEP regenerating and decarboxylating enzymes needed to operate a C_4 pathway. In both species and under both growth treatments, the activity of PEPC was greater than that of RuBisCO and in plants adapted to low CO_2, PEPC:RuBisisCO ratios were 5.9 and 1.8 for *O. alismoides* and *O. acuminata* respectively. The ratio for *O. alismoides* is similar to those reported for *Hydrilla verticillata* and the ratio for *O. acuminata* is identical to that of *Egeria densa* (Table 4) both of which are regarded as C_4 aquatic plants (Bowes 2011; Casati et al. 2000). The PEPC:RuBisCO ratio of *O. alismoides* is slightly lower than in some terrestrial C_4 plants, but very similar to the single-celled C_4 plants *Borszczowia aralocaspica* and *Bienertia cycloptera* (Voznesenskaya et al. 2002; Voznesenskaya et al. 2001) (Table 4). In contrast, terrestrial C_3 plants and aquatic plants lacking a biochemical concentrating mechanism have PEPC:RuBisCO ratios substantially less than 1 (Table 4). PPDK regenerates PEP, the substrate for PEPC. In the two species of *Ottelia*, activities of PPDK were equivalent to those of PEPC and so should be able to support PEPC activity. The high activities in *Ottelia* are similar to those in terrestrial C_4 plants (Table 4) although the ratio of PPDK to PEPC in *Ottelia* is greater. Of the two potential decarboxylating enzymes, the activity of NAD-ME was 130-times greater than NADP-ME in *O. alismoides* and 340-times greater in *O. acuminata*. NAD-ME is a mitochondrial enzyme that can act as the decarboxylating enzyme in the terrestrial single-celled C_4 plants *B. aralocaspica* and *Bienertia cycloptera* (Voznesenskaya et al. 2002; Voznesenskaya et al. 2001) (Table 4). The apparent decarboxylation by NAD-ME in *Ottelia*, if confirmed, would be the first report of an aquatic plant belonging to the NAD-ME C_4-subtype. Casati et al. (Casati et al. 2000) assumed that NADP-ME was the decarboxylation pathway in *Egeria densa* as the activity of this enzyme increased on transfer to low CO_2 conditions. However, NAD-ME actitvity was not measured and the activity of NADP-ME was about half that of PEPC so it is not impossible that NAD-ME is also involved in decarboxyation in this species. *H. verticillata* is also assumed to belong to the NADP-ME sub-group (Bowes 2011; Bowes et al. 2002) but much more evidence is available to support this contention since physiological characteristics changed in parallel to NADP-ME activity and oxygen inhibition measurements are consistent with high concentrations of CO_2 being generated in the chloroplast where NADP-ME is located (Magnin et al. 1997; Reiskind et al. 1997). In *H. verticillata*, the ratio of NAD-ME to NADP-

ME is about five, much less than that found in the two species of *Ottelia* studied here (Table 4). However, further work is needed to confirm that *Ottelia* is operating NAD-ME C_4 photosynthesis. It has been reported that versions of the enzymes used in the variants of C_4 photosynthesis can occur in C_3 plants. For example, in the C_3 *Arabidopsis* there are one or more isoforms of PEPC, PEPCK, NAD-ME, NADP-ME and PPDK which have different functions (Aubry et al. 2011). Analysis of enzyme activity might help to temper future claims of C_4 photosynthetic metabolism based solely on genomics or transcriptomics and detailed studies of biochemical turnover using short-term labelling with ^{14}C-labelled inorganic carbon should be investigated in the future.

Preliminary examination of leaf sections for both species under the light microscope has shown no evidence for Kranz anatomy (data not shown) so it is possible that *Ottelia* is also operating a single-cell C_4 mechanism. However, unlike *Hydrilla* and *Egeria*, the leaves of both *Ottelia* species are four cells thick, so RuBisCO and PEPC could be localized in different types of cell.

The C_4 system in *O. alismoides* and *O. acuminata* is not abolished at high CO_2 (400 µmol CO_2 L^{-1}; over 30-fold air-equilibrium) unlike in the two other well-studied C_4 freshwater macrophytes, *E. densa* and *H. verticillata*. The C_4 syndrome may be constitutive in *Ottelia* as it is in the marine macroalga *Udotea flabellum* (Reiskind and Bowes 1991; Reiskind et al. 1988) although the effect on *Ottelia* of other environmental factors such as low temperature or light has not been tested.

Both species of *Ottelia* studied here showed an ability to use bicarbonate as an exogenous carbon source based on the pH-drift experiments and also the rates of oxygen evolution as a function of inorganic carbon concentration, despite the use of a buffer to maintain constant pH. Bicarbonate use is a widespread feature in freshwater angiosperms (Maberly & Madsen 2002), however, its combination in a species able to show CAM has not been reported before as far as we are aware.

Distribution of biochemical CCMs in terrestrial and aquatic plants

Several lines of evidence show that in the terrestrial environment, C_4 photosynthesis became widespread around 11 to 5 million years ago during periods of hot and arid conditions and that it is

polyphyletic and arose at least 62 times (Sage et al. 2011). C_3-C_4 metabolism has been described in several species in the genera *Moricandia*, *Panicum* and *Flaveria*. C_3-C_4 intermediates are important because they are viewed as possible evolutionary intermediates between the C_3 and C_4 photosynthetic pathways (Peisker 1996). In all known *Flaveria* C_3-C_4 intermediates, both RuBisCO and PEP carboxylase are not entirely compartmentalized between mesophyll and bundle-sheath cells, as is observed in C_4 species (von Caemmerer 2000). Different *Flaveria* C_3-C_4 intermediates fix between 15 to 85% of atmospheric CO_2 into C_4 acids during short-term exposure to $^{14}CO_2$; however, transfer of label to the C_3 cycle does not occur at the rates normally observed in C_4 species (Monson et al. 1986). Our results showed that there was a statistically significant difference between light and dark acidity levels in *O. alismoides* at low CO_2 of about 24-34 μequiv g^{-1} FW in July and August. However, in September there was no indication of a diel acidity change in either CO_2 treatment. Even in the absence of a diel change in acidity, there was a substantial amount of acidity on all measuring occasions, at the end of the dark period. C_3-C_4 intermediate photosynthesis could be a possible metabolism involving organic acids besides CAM.

Aquatic C_4 photosynthesis is probably more ancient than that of terrestrial C_4 and is also likely to be polyphyletic. The marine macroalga *Udotea flabellum* (Chlorophyta, Udoteaceae) performs C_4 metabolism but PEPCK is believed to carry out the dual role of carboxylation and decarboxylation (Reiskind and Bowes 1991). It has recently been proposed that another marine macroalga *Ulva prolifera* (Chlorophyta, Ulvophyceae) has C_4 metabolism based on the presence of PEPC and PPDK (Xu et al. 2012). Within microalgae, C_3-C_4 metabolism has been described in some marine diatoms (Bacillariophyta, that arose about 180 million years ago) such as *Thalassiosira weissflogii* (Reinfelder 2011; Reinfelder et al. 2000) but appears to be absent in others such as *T. pseudonana* and *Phaedodactylum tricornutum* (Haimovich-Dayan et al. 2013; Roberts et al. 2007).

Within aquatic angiosperms, C_4 photosynthesis appears to be largely restricted to the Hydrocharitaceae, a monocotyledonous family of 18 genera and about 120 species that is believed to have an Oriental origin about 65 million years ago (Chen et al. 2012). The genus *Stratiotes* is believed to be the first diverging lineage of the Hydrocharitaceae and two clades have been recognized: Clade

A includes *Hydrilla*, *Najas*, *Vallisneria* and the seagrasses *Halophila*, *Thalassia* and *Enhalus*, and Clade B includes *Ottelia*, *Egeria*, *Elodea* and *Lagarosiphon* (Chen et al. 2012). Both clades contain species with C_4 activity: within Clade A in *Hydrilla* (Bowes 2002, 2011) and possibly in the seagrass *Halophila* (Koch et al. 2013); within Clade B in *Egeria* (Casati et al. 2000) and *Ottelia* (this study). Outwith the Hydrocharitaceae, although within the order Alismatales, the aquatic angiosperm (seagrass) *Cymodocea* (Cymodoceaceae) may also have some evidence for C_4 metabolism (Koch et al. 2013) but this requires further investigation.

There is also a high incidence of CAM-like activity, or at least evidence for elevated concentrations of organic acids, in the Hydrocharitaceae. The report here of CAM in *O. alismoides* at low CO_2 contrasts with the data from (Webb et al. 1988) where a diel change of only 7 μequiv g^{-1} FW has been found in the amphibious *Ottelia ovalifolia*; however our results suggest that CAM is facultative in *O. alismoides* and apparently absent in *O. acuminata* so this is not necessarily contradictory. *Vallisneria americana* and *V. spiralis* (Clade B) show evidence for CAM with diel changes in acidity of up to 42 and 51 μequiv g^{-1} FW respectively (Keeley 1998; Webb et al. 1988). Diel changes in acid contents in other Hydrocharitaceae such as species from the genera *Egeria*, *Elodea* and *Lagarosiphon* are relatively low (Keeley 1998; Webb et al. 1988). Earlier studies reported fixation of ^{14}C into C_4 acids in species of freshwater Hydrocharitaceae within the genera: *Egeria*, *Elodea* and *Lagarosiphon* (Brown et al. 1974; Browse et al. 1977; Degroote and Kennedy 1977; Salvucci and Bowes 1983) and also in the marine *Halophila* (Beer 1989) although there is little evidence for turnover in pulse-chase experiments. Thus, the precise role of these acids and their relationship to C_4, CAM and C_3-C_4 intermediates or other functions such as pH-regulation remains to be elucidated within the Hydrocharitaceae in species that do not appear to operate C_4 or CAM.

Comparison of *O. acuminata* and *O. alismoides*

O. alismoides is an annual plant and is widespread in tropical and warmer regions of Asia and Australia (Cook and Urmikonig 1984). It can grow in still or flowing water to a depth of about 1 m. It is also found elsewhere as a non-native such as in Louisiana in the south of the USA

(http://plants.usda.gov). In contrast, *O. acuminata* is a perennial with a restricted distribution, being confined to western China where it grows in still and flowing water to a depth of 5 m. Both species are able to use bicarbonate in addition to CO_2 as an inorganic carbon source for photosynthesis, but *O. alismoides* appears to have greater flexibility in its CCMs with apparently facultative CAM and constitutive C_4 metabolism. It is tempting to suggest that this flexibility of CCM operation may be linked to its annual growth cycle with the requirement to produce seeds at the end of a growing season, its distribution in shallow tropical waters where ecological success is likely to be favoured by high growth rates, and also that an efficient and effective carbon uptake system may increase its potential to invade other non-native habitats.

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Figure captions

Fig. 1 Comparison of the activity of C_3 and C_4 metabolic enzymes in crude extracts from *O. alismoides* (light grey bars) and *O acuminata* (dark grey bars) grown under low and high CO_2 concentrations for RuBisCO: ribulose 1,5-bisphosphate carboxylase-oxygenase (A); PEPC: PEP carboxylase (B); Ratio of PEPC to RuBisCO activity (C); PPDK: pyruvate phosphate dikinase (D); NADP-ME: NADP-malic enzyme (E); NAD-ME: NADP-malic enzyme (F). Means and standard deviation are presented. Statistical differences between high and low CO_2 treated plants are designated as follows: NS, not significant, *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$

Fig. 2 Correlations between activities of A, PPDK; B, NADP-ME and C, NAD-ME and activity of PEPC for *O. alismoides* (❍) and *O. acuminata* (●) grown at low and high concentrations of CO_2. Each point represents the mean activity from one tank. 1:1 activity is represented by a solid line

Fig. 3 Acidity of extracts from *O. alismoides* (white bars) and *O. acuminata* (grey bars) grown under low and high CO_2 concentrations, measured at the end of the dark period (hatched bars) and in the light (open bars) in July (A), August (B) and September (C). Means and standard deviation are presented. Statistical differences between high and low CO_2 treated plants are designated as follows: NS, not significant, *, $P < 0.05$. Where there is a significant difference between light and dark acidity, the difference is given as Δ

Fig. 4 Rate of net photosynthesis as a function of the concentration of HCO_3^- (A and C) or CO_2 (B and D) in *O. alismoides* (A and B) and *O acuminata* (C and D) grown under low (❍) and high (●) CO_2 concentrations

Table 1 Conditions in the two growth treatments. Mean values are given with ranges in parentheses

Conditions	Low CO_2	High CO_2
Temperature (°C)	29 (27-31)	29 (27-31)
pH[a]	8.27 (7.43-9.19)	6.99 (6.71-7.37)
Alkalinity (mequivalent L^{-1})	1.21 (0.82-1.74)	2.08 (1.94-2.23)
CO_2 (µmol L^{-1})	5 (0.1-19)	401 (156-748)
HCO_3 (mmol L^{-1})	1.05 (0.37-1.69)	1.98 (1.66-2.23)

[a]calculated as a geometric mean.

Table 2 Characteristics of the *Ottelia* species grown at low and high CO_2 concentration

Species	Chla+b / FW (mg g^{-1})		Chla : Chlb		Protein/ FW (mg g^{-1})		Specific Leaf Area (1-sided cm^2 g^{-1} FW)	
	Low	High	Low	High	Low	High	Low	High
O. acuminata	1.26	0.74	2.74	3.03	2.11	1.97	95.5	72.2
	(0.14)	(0.12)	(0.19)	(0.38)	(0.10)	(0.10)	(26.2)	(16.2)
O. alismoides	0.95	0.65	2.75	2.90	1.62	1.34	100.5	85.5
	(0.32)	(0.36)	(0.08)	(0.08)	(0.22)	(0.21)	(7.9)	(22.1)

Table 3 Carbon concentrations at the end of the pH drift experiments. Mean values are given with SD in parentheses

Species	$[CO_2]$ treatment	Starting Alk (mM)	Final Alk (mM)	Max pH	C_T (mM)	CO_2 (nM)	HCO_3^- (mM)	C_T /Alk
O. acuminata	Low	0.1	0.36 (0.13)	10.21 (0.13)	0.117 (0.056)	7.6 (2.1)	0.060 (0.024)	0.32 (0.05)
	Low	1.0	1.04 (0.01)	10.72 (0.01)	0.278 (0.011)	3.0 (0.3)	0.074 (0.004)	0.27 (0.01)
	High	0.1	0.17 (0.11)	9.64 (0.30)	0.084 (0.042)	28.9 (17.8)	0.062 (0.024)	0.53 (0.11)
	High	1.0	1.06 (0.06)	10.68 (0.02)	0.322 (0.025)	4.1 (0.4)	0.091 (0.007)	0.30 (0.01)
O. alismoides	Low	0.1	0.31 (0.13)	10.01 (0.28)	0.119 (0.047)	15.8 (13.0)	0.071 (0.035)	0.4 (0.13)
	Low	1.0	0.84 (0.06)	10.64 (0.04)	0.224 (0.018)	3.4 (0.60	0.068 (0.007)	0.27 (0.02)
	High	0.1	0.37 (0.18)	10.01 (0.21)	0.175 (0.121)	26.4 (30.6)	0.111 (0.089)	0.45 (0.15)
	High	1.0	0.83 (0.01)	10.52 (0.06)	0.290 (0.035)	7.2 (2.5)	0.107 (0.022)	0.35 (0.04)

Table 4 Comparison of activities of photosynthetic enzymes in aquatic and terrestrial plants. Activities (µmol mg^{-1} Chl*a* h^{-1}) were measured between 22 and 30°C

[*] Adapted to low CO_2 conditions.

ENVIRONMENT/ Species	Type	RuBisCO	PEPC	PPDK	NADP-ME	NAD-ME	PEPC: RuBisCO	Reference
AQUATIC								
14 species	C_3	187	29	-	-	-	0.2	(Farmer et al. 1986)
Hydrilla verticillata[*]	C_4 NADP-ME	23-45	116-330	30-41	23-30	104-175	6.6[$]	(Holaday and Bowes 1980; Magnin et al. 1997; Salvucci and Bowes 1983)
Egeria densa[*]	C_4 NADP-ME	71	130		60		1.8	(Casati et al. 2000; Salvucci and Bowes 1983)
Eleocharis								(Ueno et al. 1988)
Isoetes howellii	CAM	256	36	110	37	2	0.1	(Keeley 1998)
Crassula aquatica	CAM	392	178	208	78	2	0.5	(Keeley 1998)
Ottelia acuminata[*]	C_4?	55	100	114	11	3740	1.8	This study
Ottelia alismoides[*]	C_4 NAD-ME/CAM?	45	264	246	13	1740	5.9	This study
TERRESTRIAL								
Suaeda heterophylla	C_3	424	18	15	nd	168	0.04	(Voznesenskaya et al. 2001)
Borszczowia aralocaspica	Single-cell C_4	130	768	511	145	226	5.9	(Voznesenskaya et al. 2001)
Bienertia cycloptera	Single-cell C_4	258	1368	101	20	510	5.3	(Voznesenskaya et al. 2002)
Average	C_4 NADP-ME	60-240	780-1440	240-480	600-960	<60	7.4[$]	(Kanai and Edwards 1999)
Average	C_4 NAD-ME	60-180	720-1500	24-540	<60	300-1080	9.3[$]	(Kanai and Edwards 1999)
Average	C_4 PEP-CK	60-240	1020-1620	120-240	<60	60-180	7.3[$]	(Kanai and Edwards 1999)
Mesembryanthemum crystallinum	CAM	80	1039	44	62	112	13.0	(Winter et al. 1982)

[$] Ratio based on average of the range of activities for PEPC and RuBisCO.

nd, Not determined.

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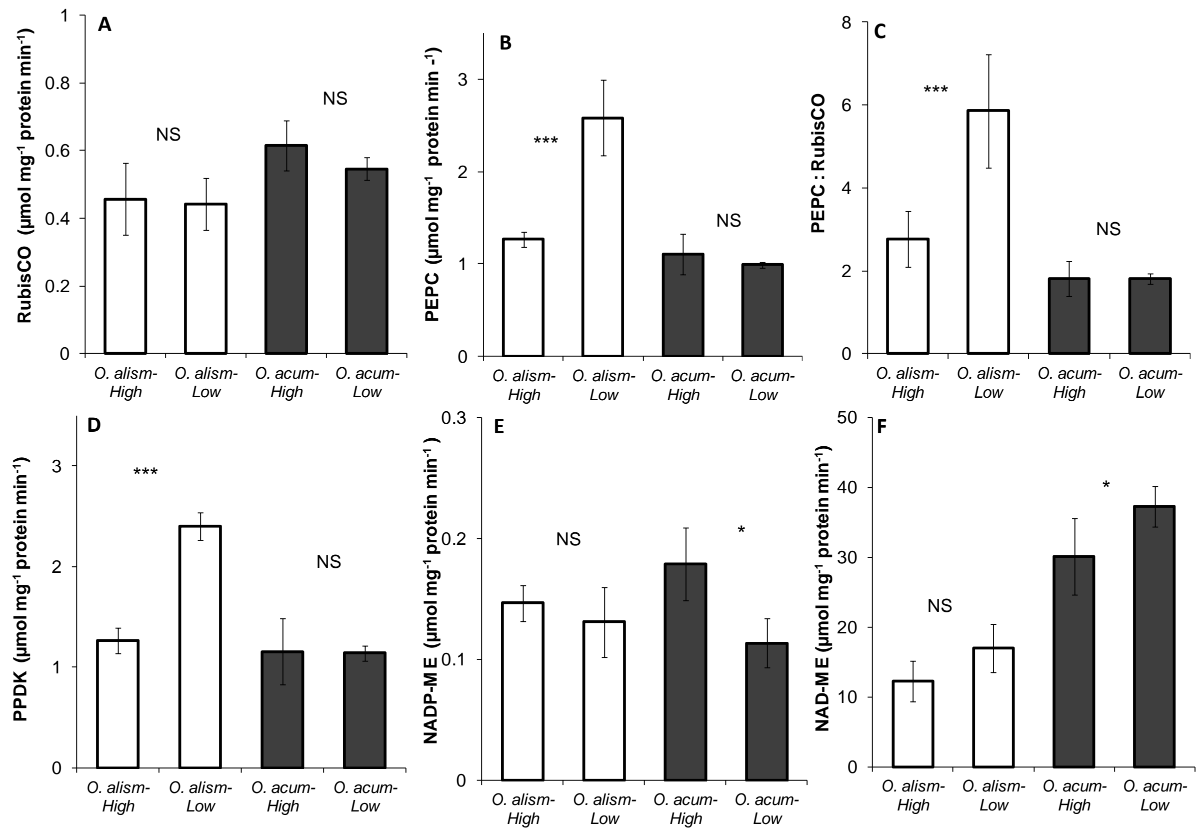

Figure 1.

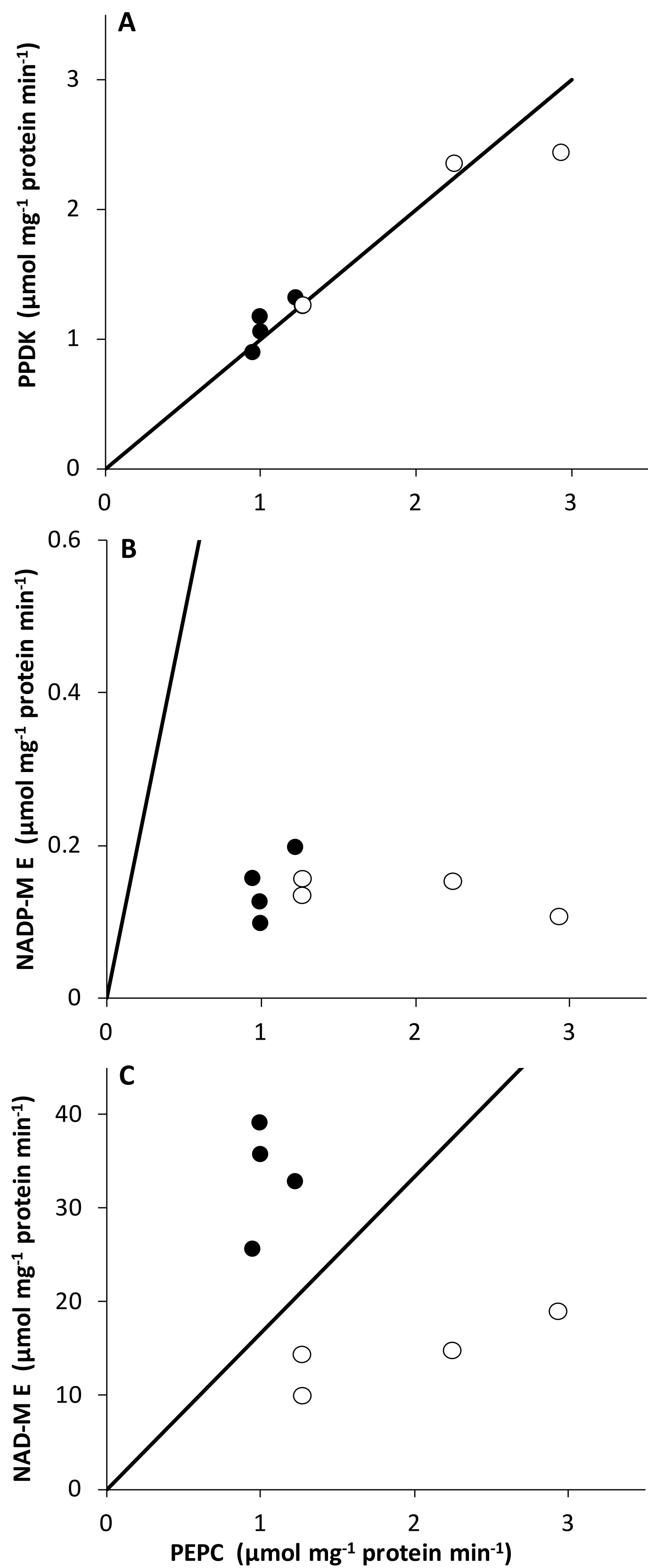

Figure 2.

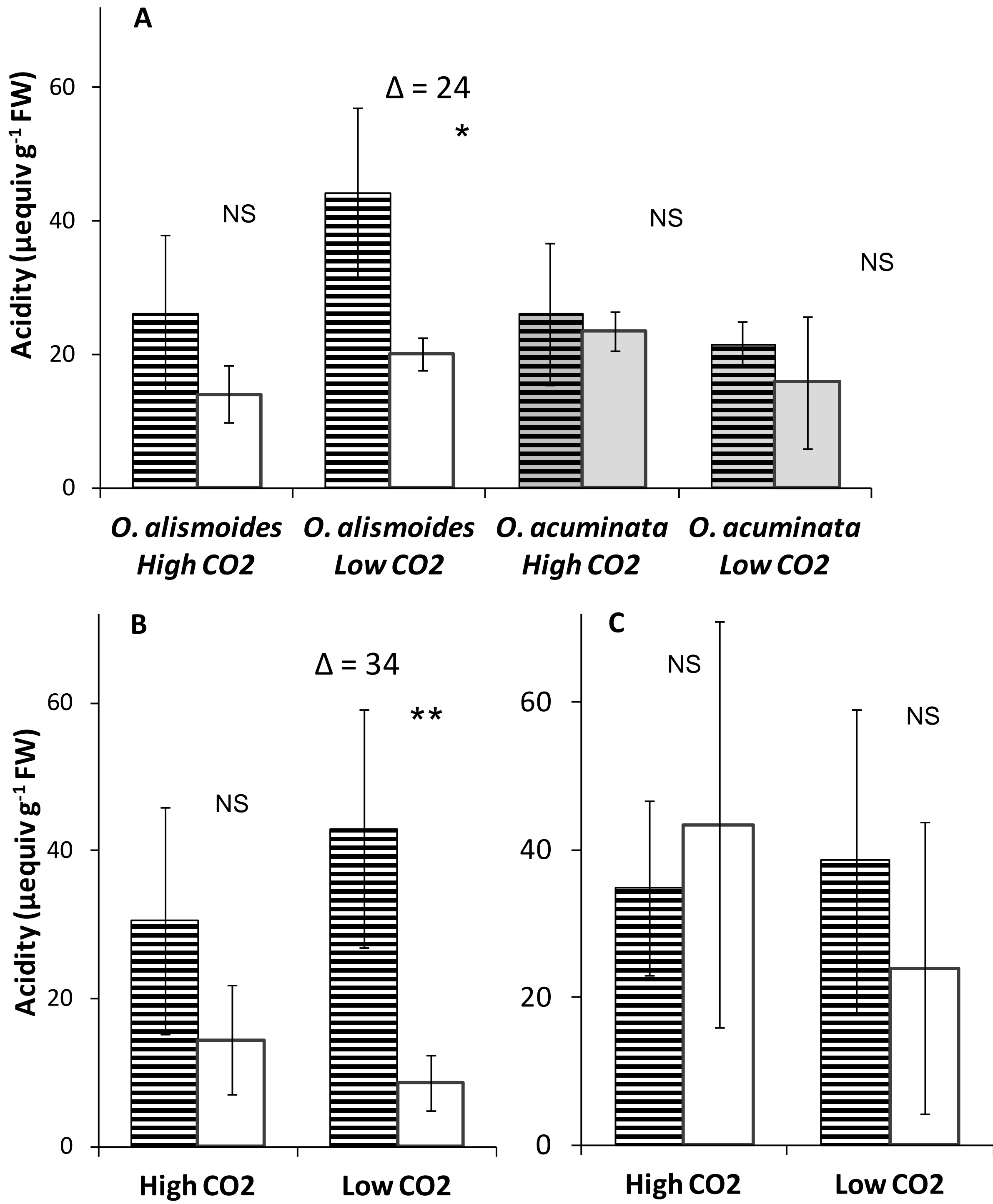

Figure 3.

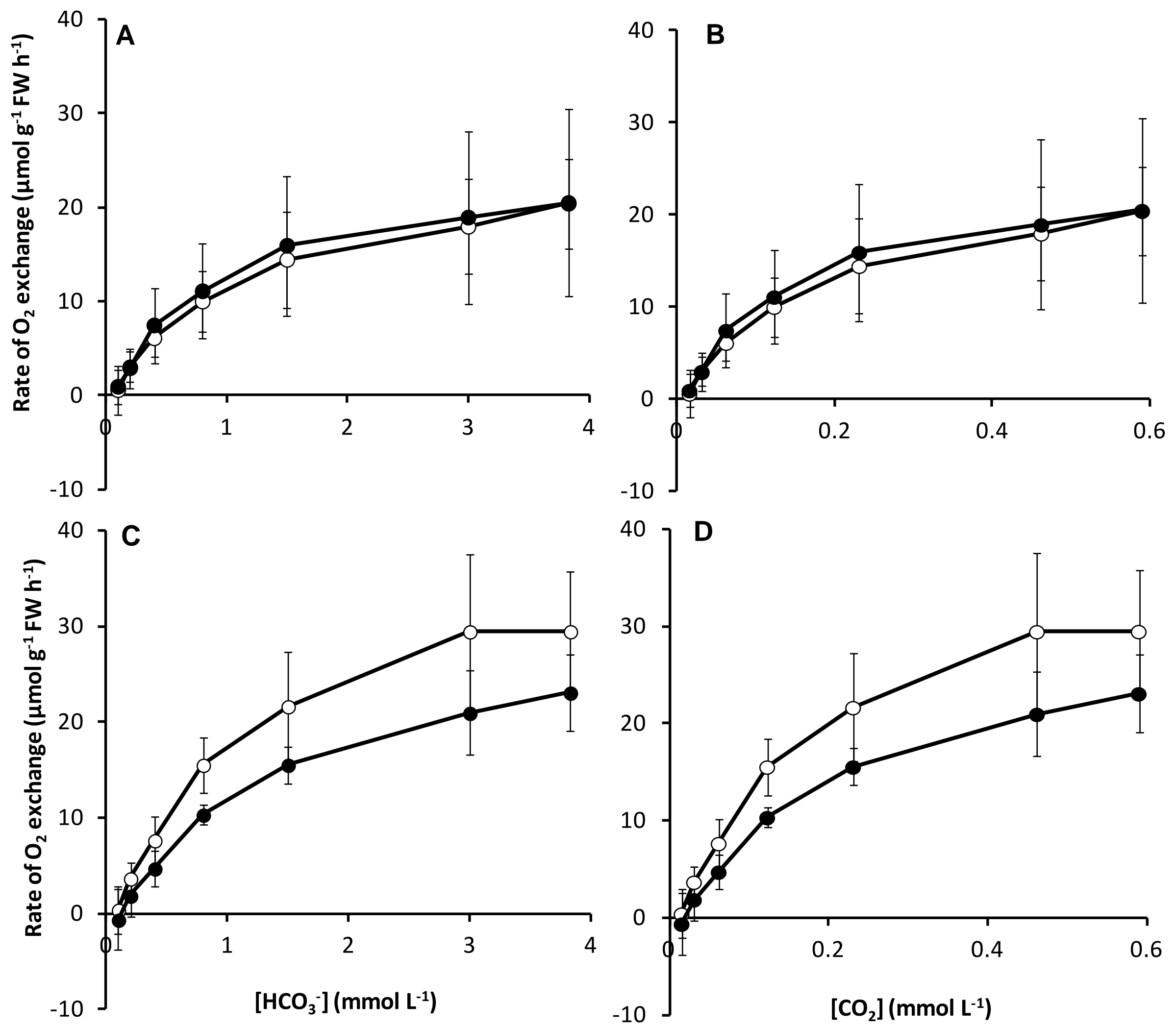

Figure 4.