Responses of Leaf Anatomy and CO2 Concentrating Mechanisms of the Aquatic Plant *Ottelia cordata* to Variable CO2

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Acclimation to variable CO2 was studied in floating leaves of the freshwater monocot *Ottelia cordata* grown in either low or high CO2. The most striking anatomical variations responding to high CO2 included the enlarged upper epidermal cells and the decreased area of epidermal chloroplasts. Stomata that distributed on the upper surface, and the stomatic chamber area, showed no significant response to high CO2. pH-drift experiments indicated that floating leaves of *O. cordata* were able to use bicarbonate regardless of CO2 concentrations. Photosynthetic enzyme activities and patterns of organic acids fluctuation confirmed that floating leaves of *O. cordata* can operate CAM only at low CO2, and perform C4-like metabolism at both high and low CO2. Overall, the present results imply that the floating leaves of *O. cordata* does not just rely on the atmospheric CO2 for its inorganic carbon, but is also dependent on CO2 and bicarbonate in the water. By showing these effects of CO2 variation, we highlight the need for further experimental studies on the regulatory mechanisms in *O. cordata* floating leaves, that prevent futile cycling among the three CO2 concentrating mechanisms (bicarbonate use, C4, and CAM metabolism) and the strategy for exploiting atmospheric CO2, as well as studies on the detailed biochemical pathway for C4 and CAM metabolism in this species.

Keywords: CO2 availability, C4, crassulacean acid metabolism, bicarbonate use, CO2 concentrating mechanisms, organic acids

INTRODUCTION

Since preindustrial times, the atmospheric CO2 concentration has increased from ~280 ppm to currently over 400 ppm and is predicted to reach 800 ppm by the year 2100 (IPCC, 2014). The increasing concentration of atmospheric CO2 might influence plant traits, such as growth, photosynthesis, as well as the morphology and anatomy of leaves (Long et al., 2004). The responses of terrestrial plants to increasing atmospheric CO2 have been studied extensively (e.g. Long et al., 2004; Ainsworth and Long, 2005), however, studies on aquatic plants are sparse and mainly focus on marine species (Johnson et al., 2013; Koch et al., 2013). The effects of increasing...
atmospheric CO₂ on freshwater macrophytes appear to be more intricate and might be species-and water body-specific (Hussner et al., 2019). Increasing the atmospheric CO₂ will increase the availability of dissolved inorganic carbon (DIC) and decrease the pH in some aquatic ecosystems; this will also increase the proportion of CO₂ and HCO₃⁻ within the pool of DIC, within a given pH range (Pedersen et al., 2013). Nevertheless, in some cases such as the productive lakes, the aquatic ecosystems are pulled out of the air-equilibrium either by rapid photosynthesis that decrease CO₂ concentrations or rapid decomposition of litter in the sediment that increase CO₂ concentrations in the water. This can generate die changes in CO₂ concentrations of over 100-fold as well as the seasonal changes (Maberly, 1996; Schippers et al., 2004). Generally, photosynthesis in submerged aquatic plants is more sensitive and responsive to increasing CO₂ than HCO₃⁻ (Maberly and Madsen, 1998).

As to photosynthesis in submerged aquatic plants, CO₂ is the preferred form of carbon and a number of species could only use CO₂. However, about 50% of tested species have evolved strategies to use HCO₃⁻ to handle CO₂ limitation (Iversen et al., 2019). The problem of limited inorganic carbon supply is caused by several factors (Maberly and Gontero, 2017): (1) the CO₂ diffusion rate in water is ~10 000 times lower than in air, which hinder the CO₂ transport into freshwater plants through the boundary layer; (2) when the requirement for inorganic carbon by photosynthesis exceeds the supply from the environment, especially in productive systems, the CO₂ can be depleted and its concentration close to zero. Thus, in addition to HCO₃⁻ use, freshwater plants have evolved a diversity of CO₂ concentrating mechanisms (CCMs) to increase further the efficiency of carbon utilization. Although much less widespread than HCO₃⁻ use, some freshwater macrophytes possess a type of C₄ (Bowes, 2011) or crassulacean acid metabolism (CAM)-like metabolism (Keeley, 1998). Both C₄ and CAM implement primary carbon fixation via the enzyme phosphoenolpyruvate carboxylase (PEPC), that is active during either day for C₄ or during night for CAM. Unlike terrestrial C₄ plants, in aquatic species, C₄ metabolism can be induced under the limitation of inorganic carbon, or be constitutive independent of the CO₂ concentration (Maberly and Gontero, 2017). The frequency of aquatic species with CAM is ~9% (Maberly and Gontero, 2018), and the utilization of CO₂ can occur during daytime, allowing the freshwater CAM plants to assimilate CO₂ continuously (Keeley, 1998; Madsen et al., 2002), which differs from the terrestrial CAM plants. CAM metabolism is a plastic process in freshwater plants (Bowes and Salvucci, 1989), and CO₂ concentration can affect its activity (Klavsen and Maberly, 2010; Zhang et al., 2014). Except the amelioration strategies described above, exploitation strategies are another diversity of strategies to minimize the carbon constraint in aquatic plants that involve morphological and anatomical features to give access to higher availability of free CO₂ from the sediment or the atmosphere (Klavsen et al., 2011; Maberly and Gontero, 2018). Floating and aerial leaves of some amphibious species are the examples with exploitation strategies that could exploit more CO₂ from the atmosphere (Sand-Jensen et al., 1992; Sand-Jensen and Frost-Christensen, 1998). The compilation of CCMs in aquatic macrophytes showed that less than 25% species with an ability to use HCO₃⁻ have alternative strategies, which mainly comprise C₄ and access to atmospheric CO₂; nevertheless, CAM and C₄ do not usually coexist in one species (Maberly and Madsen, 2002; Maberly and Gontero, 2017). The submerged freshwater plant, Ottelia alismoides, is hitherto the only known species to operate three CCMs (HCO₃⁻ use, C₄ and CAM metabolism) in the same tissue (Zhang et al., 2014; Shao et al., 2017).

In addition to the responses of CCMs to variable CO₂ availability, the size and anatomical structure of plant leaves are also influenced by CO₂ concentration (Radoglou and Jarvis, 1990). Enhanced CO₂ generally increases the size of terrestrial plants (Pritchard et al., 1999). In Brassica juncea, high CO₂ increased the thickness of the upper and lower epidermis, as well as the mesophyll cells of leaves (Upreyt et al., 2001). While, in sorghum, the increase in growth CO₂ resulted in a marked decrease in the thickness of the bundle sheath (Watling et al., 2000). In O. alismoides, high CO₂ increased the thicknesses of the epidermal layers, the air space and mesophyll cells (Han et al., 2020). To our knowledge, there is little information in the literature concerning the effects of varying CO₂ availability on leaf structure and the relationships between structure and function in aquatic plants. Ottelia cordata (Wallich) Dandy, a member of the Hydrocharitaceae, is an aquatic macrophyte distributed in China, Cambodia, Myanmar and Thailand (Wang et al., 2019; Li et al., 2020). It is a perennial and heterophyllous plant with linear or lanceolate submerged leaves and ovate-cordate floating leaves. Cultivation experiments showed that during its early growth phases, O. cordata is totally submerged and it only grows submerged leaves in the first year; at the second year and after, it only grows floating leaves, which is the only part of the plant that comes into contact with air (He and Sun, 1990). It was shown recently that O. cordata floating leaf is a HCO₃⁻ user and perhaps operate CAM (Cao et al., 2019). The aim here was to quantify the extent of plasticity of CCMs and leaf anatomy in this species, in response to varying CO₂ availability in the water, to evaluate further possible effects of CO₂ enrichment on this species.

MATERIALS AND METHODS

Plant Material

Healthy O. cordata plants were collected randomly from a wild population at Haikou, Hainan Province, China. After collection, in order to eliminate the differences caused by environmental heterogeneity, the plants were cultured in plastic pots with aseptic soil and filled with water, and were illuminated with FSL T5/865 28 W fluorescence tubes in a growth room located in Wuhan Botanical Garden, with ambient temperature (13°C–17°C) and ~130 μmol photon m⁻² s⁻¹ (Li-Cor underwater sensor, UWQ, connected to a Li-Cor LI-1400 data logger) at the water surface, with a 14-h light (08:00–22:00)/10-h dark photoperiod. The light intensity condition
was chosen as a trade-off between having sufficient light for photosynthesis to avoid the effects induced by low light levels on anatomy and photosynthetic physiology, and not so much light to avoid causing photodamage when at low CO₂ concentrations. The water depth was maintained at ~30 cm during the acclimation. The transplanted plants were cultured for 3-4 weeks before they were used in the experiments.

### Response of O. cordata Floating Leaves to Different CO₂ Concentrations

In the different CO₂ acclimated experiments, the pots of O. cordata plants were put into the white plastic tanks (65 cm × 45 cm × 35 cm) containing tap water and incubated at two CO₂ concentrations as described previously (Huang et al., 2018). In the high CO₂ treatment (HC), the target pH of 7.0 was maintained between 6.52 and 7.06 by bubbling the medium with pure CO₂ under the control of a microcomputer pH controller (model 6311, Jenico Instruments, USA), producing CO₂ concentrations between 380-1603 µM with a mean of 941 µM. A low CO₂ (LC) concentration was produced by the natural photosynthesis of the plants which depleted the inorganic carbon of the water, and increased pH from 8.09 to 8.91, with a CO₂ concentration range of 3-28 µM and a mean of 15 µM. The acclimation of different CO₂ with four replicate pots per treatment, lasted four weeks in the growth room. Over the whole period of acclimation, alkalinity, pH and temperature were measured to calculate the CO₂ concentration using the equations reported in Maberly (1996). More information of the conditions in the treatments is shown in Table 1. Following the different CO₂ acclimation, there were newly floating leaves emerged, which were harvested for anatomical structure observation and physiological parameters measurement.

### Leaf Anatomy Observation by Light Microscopy and Transmission Electron Microscopy

The youngest fully developed floating leaves in both CO₂ treatments were sampled at 19:00 and were completed within half an hour. Then the leaves were sliced into 3 mm × 3 mm leaf fragments along the midrib. The segments were fixed immediately in 2.5% glutaraldehyde (pH 7.4) at 4°C overnight and then post-fixed in 1% OsO₄ at 4°C for 2.5 h. The semithin sections and ultrathin sections of the leaf samples were obtained according to the method of Han et al. (2020). Semithin sections were stained with methylene blue and observed using a digital light microscope (Motic BA310). Quantitative characteristics of leaf structures including the area and size of the epidermal and mesophyll cells, as well as the air space and stomatic chamber, were measured using the Motic Images Plus 2.0 ML software. Ultrathin sections were examined and photographed under a TEM (HT7700, Hitachi, Japan). The area and size of chloroplasts and starch grains in the electron micrographs were measured with ImageJ software. At least 10 epidermal/mesophyll cells from both the upper and lower surfaces were measured per leaf from the digitized images.

### pH-Drift Experiments and Drift Parameters

The ability of O. cordata floating leaves to use HCO₃⁻ was assessed in pH-drift experiments (Maberly and Spence, 1983). About 0.2-0.3 g fresh weight (FW) of leaves collected from different CO₂ acclimated plants, rinsed in clean tap water and then incubated in 70-ml test bottles with 50 ml of test solution (equimolar concentrations of NaHCO₃ and KHCO₃ at an overall concentration of 1 mM). The bottles with plant leaves were sealed with glass stoppers and incubated in a growth room at 25°C and ~130 µmol photon m⁻² s⁻¹ PAR. After ~24 h continuous irradiance, the final pH of the medium was measured with a pH electrode (model IP-600-9 Jenico Instruments, USA) connected to a microcomputer pH controller. The final alkalinity of the solution was measured by Gran titration (Zhang et al., 2014). The total Ci remaining at the end of pH-drift, which represents the dissolved inorganic carbon comprising free CO₂, HCO₃⁻ and CO₃²⁻, was designated as Cᵣ. The concentration of Cᵣ, CO₂ and HCO₃⁻ in the solution were calculated from the alkalinity, temperature and pH (Maberly, 1996). The quotient of Cᵣ/alkalinity reflects the ability of O. cordata floating leaves to deplete inorganic carbon (Maberly and Spence, 1983).

### Photosynthetic Enzyme Activity Measurement

Floating leaves were harvested at 21:00 h (dusk) before the lights turn off and at 07:00 h (dawn) before the lights turn on and immediately frozen in liquid nitrogen for further measurement of photosynthetic enzyme activity. The activity of ribulose 1,5-bisphosphate carboxylase–oxigenase (Rubisco), phosphoenol pyruvate carboxylase (PEPC), pyruvate phosphate dikinase (PPDK) and NADP-malic enzyme (NADP-ME), were determined according to the methods described in Zhang et al. (2014), and were assessed from the rates of NADH disappearance or appearance at 340 nm and 25°C, using a microplate reader (Tecan M200 PRO, Austria).

### CAM Activity and Organic Acids Content

Fresh O. cordata floating leaves (0.2–0.5 g), were collected at 21:00 h (dusk) and 07:00 h (dawn) and immediately frozen in liquid nitrogen for further measurement of acidity and organic acids. The acidity was measured according to the method of Zhang et al. (2014).

The organic acids in O. cordata floating leaves were extracted and detected based on the methods described previously (Pedersen et al., 2011). About 0.2 g frozen leaves were ground and extracted with ice-cold 5% (v/v) perchloric acid using a mortar and pestle. The homogenized samples were centrifuged at 8,000 g for 10 min, the supernatant was collected and the
procedure was repeated once with ice-cold 5% perchloric acid. The supernatants were mixed and adjusted to pH 3.0–3.5 using saturated K₂CO₃ solution, and then centrifuged again at 4,000g for 5 min. The resulting supernatant was filtered through a 0.22-

μm filter and transferred to vials for quantification with high performance liquid chromatography (HPLC). The component of organic acids in the extraction was separated in an Athena C18-WP (4.6 mm × 250 mm) column (CNW Technologies GmbH, Germany) maintained at a constant temperature of 30°C. The mobile phase consisted of 25 mM KH₂PO₄ (pH 2.5) with methanol at a constant flow rate of 0.8 ml min⁻¹. The concentration of organic acids was detected at 220 nm with a photodiode array detector (PDA) coupled to a HPLC (Waters e2695 system, Milford, MA, USA). The quantitative determination of the organic acids was obtained by analyzing the chromatographic data using Data System based on the corresponding retention time and peak area of the different organic acids.

**Chlorophyll, Dry Weight, and Leaf Area**

The content of chlorophyll a and b in *O. cordata* floating leaves were determined spectrophotometrically at 649 and 665 nm (TU-1810PC, Purkince General, China) according to the method described in Shao et al. (2017). Dry weight (DW) was measured after the leaves were dried for 48 h at 80°C. Projected (1-sided) leaf area was calculated from digital photographs with AreaAna software (Huazhong University of Sciences and Technology, China).

**Statistical Analysis**

The data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, USA). The significance of CO₂ treatment and sample time were determined with two-way ANOVA. The independent sample t-tests were used for the comparisons on the effects of CO₂ concentrations on the morphological and anatomical characteristics as well as the pH-drift traits. Pearson correlation was used to test the correlation between different enzyme activities and the significance level was set at 5%.

**RESULTS**

**Chlorophyll, FW/DW, and Leaf Area**

High CO₂ did not have a statistically significant effect on the chlorophyll content, FW/DW and leaf area in *O. cordata* floating leaves, when compared with that of low CO₂ (p>0.05; **Table 2**).

**Anatomical Structure and Ultrastructure of Chloroplast**

The leaf anatomy of *O. cordata* floating leaves under high and low CO₂ were shown in **Figure 1** and the basic structure of leaves did not vary with CO₂ concentrations. The surface of *O. cordata* floating leaf was relatively flat and smooth. Transverse section observation showed that the upper and lower epidermis was single-layered rectangular cells, closely arranged (**Figures 1A–F**). The area and width of the upper epidermal cells in leaves grown under high CO₂ were notably larger than those under low CO₂ (p<0.05; **Table 2**), however there was no significant difference in lower epidermal cells (p>0.05; **Table 2**). The length and length/width ratio of the upper and lower epidermal cells were not significantly affected by CO₂ concentrations (p>0.05; **Table 2**). Stomata only distributed on the upper epidermis of floating leaves of *O. cordata* (**Figures 1B, E**). The mesophyll tissues were developed, and there were air spaces interspersed among the lower mesophyll cells. The differentiation between palisade tissues and spongy tissues was present (**Figures 1B, C, E, F**). There was no significant difference in the area of air spaces, stomatic chamber, upper and lower mesophyll cells between high and low CO₂ treated leaves (p>0.05; **Table 2**).

Considering chloroplasts can differ in cells responding to the variation of environmental factors, we evaluated chloroplast shape, size and ultrastructure in both epidermal and mesophyll

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**TABLE 2 | Effects of low and high CO₂ concentrations on the morphological and anatomical characteristics of the *O. cordata* floating leaves.**

| Character                          | Low CO₂          | High CO₂         |
|-----------------------------------|------------------|------------------|
| Whole leaf characteristics        |                  |                  |
| Chlorophyll a (mg/g FW)           | 1.24 (0.34)      | 1.02 (0.10)      |
| Chlorophyll b (mg/g FW)           | 0.43 (0.13)      | 0.32 (0.05)      |
| Chlorophyll a+b (mg/g FW)         | 1.67 (0.41)      | 1.34 (0.13)      |
| FW/DW                             | 9.79 (1.69)      | 9.43 (1.33)      |
| Specific leaf area (1-sided cm² g⁻¹FW) | 51.17 (1.68) | 58.97 (10.70)   |
| Cell characteristics              |                  |                  |
| Upper epidermis cell length (µm)  | 28.75 (8.27)     | 31.73 (6.82)     |
| Upper epidermis cell width (µm)   | 21.57 (2.89)     | 26.60 (4.23)     |
| Length/width of upper epidermis cell | 1.33 (0.34)  | 1.22 (0.33)      |
| Lower epidermis cell length (µm)  | 39.06 (7.62)     | 35.12 (6.53)     |
| Lower epidermis cell width (µm)   | 31.11 (4.74)     | 29.66 (4.02)     |
| Length/width of lower epidermis cell | 1.27 (0.26)  | 1.19 (0.19)      |
| Upper epidermis cell area (µm²)   | 643.52 (184.62)  | 744.73 (208.08)  |
| Lower epidermis cell area (µm²)   | 1136.8 (352.82)  | 1060.19 (193.92) |
| Upper mesophyll cell area (µm²)   | 2191.88 (3290.49) | 1370.04 (427.98) |
| Lower mesophyll cell area (µm²)   | 1027.64 (338.36) | 981.53 (313.47)  |
| Chloroplasts in mesophyll cells   |                  |                  |
| Chloroplast major axis (µm)       | 7.48 (0.82)      | 4.87 (1.11)      |
| Chloroplast minor axis (µm)       | 3.02 (0.63)      | 1.95 (0.26)      |
| Major axis/minor axis of chloroplast | 2.50 (0.25)   | 2.50 (0.25)      |
| Area of chloroplast (µm²)         | 17.01 (3.90)     | 7.29 (2.28)      |
| Area of starch (µm²)              | 0.35 (0.28)      | 0.11 (0.10)      |
| Area ratio of starch to chloroplast | 0.02 (0.01)     | 0.01 (0.01)      |
| Chloroplasts in epidermal cells   |                  |                  |
| Chloroplast major axis (µm)       | 6.86 (1.08)      | 6.81 (1.29)      |
| Chloroplast minor axis (µm)       | 2.36 (0.61)      | 2.80 (0.97)      |
| Major axis/minor axis of chloroplast | 2.66 (0.90)   | 2.66 (0.90)      |
| Area of chloroplast (µm²)         | 12.95 (5.02)     | 14.84 (6.31)     |
| Area of starch (µm²)              | 0.68 (0.74)      | 1.05 (0.63)      |
| Area ratio of starch to chloroplast | 0.04 (0.04)     | 0.08 (0.04)      |

Mean values (n=2–4) are given with s.d. in parentheses. Significant differences between leaves treated with different CO₂ concentration are shown based on independent sample t-tests. Data with different lower-case letters are significantly different between low and high CO₂ treatments (p<0.05). Data with different upper-case letters are significantly different between chloroplasts in epidermal and mesophyll cells (p<0.05).
In leaves grown at low and high CO$_2$, the epidermal chloroplasts were spindle-shaped (Figures 1G, I). The epidermal chloroplasts under low CO$_2$ (Figure 1G) had longer length for the major and minor axis, bigger size and more starch area occupied in the chloroplast, when compared with those at high CO$_2$ (Figure 1I; p<0.05; Table 2). However, the ratio of major axis length and minor axis length of the chloroplast and the area ratio of starch to chloroplast were not notably influenced by CO$_2$ concentrations (p>0.05; Table 2). For the mesophyll chloroplast, when compared with low CO$_2$ condition, except the slight but significant increased minor axis length at high CO$_2$ (p<0.05; Figures 1H, J; Table 2), there was no other significant difference on the ultrastructure of this type of chloroplast (p>0.05; Table 2). The comparison between these two types of chloroplasts showed that, at low CO$_2$, the epidermal chloroplasts had significantly larger area but lower area ratio of starch to chloroplast than mesophyll chloroplasts (p<0.05; Table 2). When at high CO$_2$, the epidermal chloroplasts had significantly smaller size and area, as well as the smaller starch area and lower area ratio of starch to chloroplast than mesophyll chloroplasts (p<0.05; Table 2).

**pH-Drift Characteristics**

The pH-drift experiments provided clear evidence for HCO$_3^-$ use in both CO$_2$ conditions in *O. cordata* floating leaves (Table 3). Both high and low CO$_2$ treated *O. cordata* floating leaves raised pH and reduced the concentration of free CO$_2$ and HCO$_3^-$ in the medium significantly from the initial values of 7.9, 23 μM and 0.99 mM. Final pH ranged from 10.33 for high CO$_2$ acclimation to 10.62 for low CO$_2$ acclimation. Correspondingly, the high and low CO$_2$ acclimated leaves depleted CO$_2$ and HCO$_3^-$ concentrations to 17 and 4 nM, 0.18 and 0.08 mM, respectively. The [CO$_2$], [HCO$_3^-$], and [C$_3$] remaining at the end of pH-drift were significantly higher at high CO$_2$ condition as compared with low CO$_2$ acclimation (p<0.05). The quotient of C$_3$/Alk, used to estimate the carbon uptake ability, was significantly lower in low CO$_2$ acclimated leaves (p<0.05), indicating that the ability of using HCO$_3^-$ was more efficient under low CO$_2$ condition.

**Key Photosynthetic Enzymes Activity**

Rubisco activity was 4.4-fold higher in low CO$_2$ compared to high CO$_2$ acclimated *O. cordata* floating leaves at dawn (p<0.05;
TABLE 3 | Final pH, alkalinity, and carbon concentrations at the end of pH-drift experiments.

| Treatments    | Final pH  | Alk   | CO₂(mM)  | CO₂/Alk  |
|---------------|-----------|-------|----------|----------|
| High CO₂      | 10.33(0.12)a | 1.04(0.11)a | 0.49(0.05)a | 0.017(0.008)a |
| Low CO₂       | 10.62(0.16)b | 1.06(0.12)a | 0.34(0.10)b | 0.000     |

Mean values (n=3–4) are given with s.d. in parentheses. *calculated as a geometric mean. Alk means alkalinity. Data with different lower-case letters within one column are significantly different between high and low CO₂ acclimated O. cordata floating leaves (p<0.05).

We also analyzed organic acids levels in floating leaves of O. cordata at dusk and dawn. The malic acid concentrations did not differ between the two time points at high CO₂ (p>0.05; Figure 3B, Table 4). In contrast, leaf tissues of low CO₂ treatment showed significant dusk/dawn oscillation in malic acid content. The concentration of malic acid at dusk was only 25% of that at dawn, showing an obvious depletion of the malate pool during day period at low CO₂ (p<0.05; Figure 3B, Table 4). However, the accumulation of malic acid at dawn did not change with the concentrations of CO₂ (p>0.05). Concentrations of citric acid, aspartic acid and trans-aconitic acid in the leaf tissue did not change between the two sampling times and CO₂ conditions (p>0.05; Figures 3C–E, Table 4). In contrast, the concentration of fumaric acid in high CO₂ acclimated O. cordata floating leaves was 5.5-fold higher at dusk and 4.1-fold higher at dawn than in low CO₂ (p<0.05), but with no significant differences between the two sampling times at both CO₂ treatments (p>0.05; Figure 3F, Table 4).

TABLE 4 | Two-way ANOVA results for physiological parameters in O. cordata, with CO₂ concentration and time as factors.

| Variables       | CO₂      | Time     | CO₂ x Time |
|-----------------|----------|----------|------------|
| Rubisco         | F 12.953 | p-value 0.007 | F 0.796    |
| PEPC            | F 30.982 | p-value 0.001 | F 0.763    |
| PEPDK/Rubisco   | F 25.209 | p-value 0.001 | F 0.030    |
| PPDK            | F 7.250  | p-value 0.027 | F 0.423    |
| NADP-ME         | F 2.013  | p-value 0.194 | F 0.836    |
| Acidity         | F 8.392  | p-value 0.016 | F 0.220    |
| Malic acid      | F 13.847 | p-value 0.006 | F 0.008    |
| Citric acid     | F 0.690  | p-value 0.430 | F 0.298    |
| Aspartic acid   | F 0.011  | p-value 0.918 | F 0.534    |
| trans-Aconitic acid | F 0.500 | p-value 0.500 | F 0.572    |
| Fumaric acid    | F 141.241 | p-value 0.000 | F 0.066    |

Significant p-values (p<0.05) are shown in bold. The degrees of freedom =1 in all cases.

DISCUSSION

Responses of Anatomy of O. cordata Floating Leaves to Variable CO₂

Through the transverse section observation of the O. cordata floating leaves, some anatomical changes were observed following acclimation with different CO₂. This is in agreement with previous reports in terrestrial or aquatic plants (Thomas and Harvey, 1983; Bray and Reid, 2002; Driscoll et al., 2006; Han et al., 2020). The most striking change occurred in O. cordata floating leaves responding to high CO₂ is the increased transverse-section area and width of upper epidermal cells. A similar increase of the area of upper epidermal cell in O. alismoides grown under elevated CO₂ was found (Han et al., 2020). In maize, the epidermal cell size from the paradermal view was also shown to be highly responsive to environmental CO₂ concentration (Driscoll et al., 2006). This would suggest that the enlarged epidermal cells are formed in response to high CO₂. This response may have come about to have consequences for photosynthesis possibly by expanding CO₂ diffusion through the upper epidermal cell membrane. Nevertheless, in some species, such as in Phaseolus vulgaris L. seedlings (Radoglo and Jarvis, 1992), the epidermal cells remained constant in size irrespective of CO₂ treatment.

Stomata were found to be distributed on the upper surface of O. cordata floating leaves. Generally, most submerged leaves do not possess stomata, or, where they do exist but they are nonfunctional (Sculthorpe, 1967). However, floating leaves of

Daily Oscillations of Acidity and Organic Acids

The CAM capacity in O. cordata floating leaves was assessed initially by measuring the diel change in acidity. Across the high and low CO₂ conditions, acidity levels varied between 35 and 89 µeqv g⁻¹ FW at dusk and between 54 and 74 µeqv g⁻¹ FW at dawn (Figure 3A). There was a significant difference between dusk and dawn acidity levels in O. cordata floating leaves at low CO₂ of about 20 µeqv g⁻¹ FW (p<0.05), in contrast there was no evidence for diel acidity variation at high CO₂.

Table 4), in contrast at dusk the difference was not statistically significant (p>0.05; Figure 2A). PEPC activity was 1.5-fold higher in low CO₂ compared to high CO₂ leaves (p<0.05; Table 4), at both dusk and dawn (Figure 2B). In high CO₂ acclimated O. cordata floating leaves, the PEPC : Rubisco ratio was 1.7 at the end of day and increased to 4.3 by the end of night. For low CO₂ conditions, the ratio was 1.1 at the end of day and increased slightly to 1.5 by the end of night (Figure 2C, Table 4). PPDK showed a similar pattern to PEPC (Figure 2D). The CO₂ concentration triggered a 1.3-fold increase in O. cordata floating leaves at low compared to high CO₂ at dusk (p<0.05), but had a slightly and not significantly promotion at dawn (p>0.05). The activity of the decarboxylating enzyme NADP-ME did not vary with the CO₂ concentrations (Figure 2E). There was a significant correlation between activity of PEPC vs. Rubisco and PPDK vs. PEPC (p<0.05; Figures 2F, G). Activity of NADP-ME did not correlate with changes in activity of PEPC (data not shown).
aquatic plants, such as the aquatic pteridophyte *Marsilea*, water lilies, and *Potamogeton octandrus* have stomata on the upper surface (Lin and Yang, 1999; Rudall and Knowles, 2013; Li et al., 2019). The stomata of terrestrial plants generally distribute more on lower epidermis than upper epidermis in order to reduce water loss. However, in aquatic plants, the distribution of stomata on the upper epidermis is obviously greater than that on the lower epidermis, since the upper surface of the floating leaf is accessible to the air. Floating leaves of aquatic plants is one of the examples with exploitation strategies to overcome the problem of carbon limitation that could exploit more constant and available CO₂ from the atmosphere based on stomata (Maberly and Gontero, 2018). Observations of the ultrathin sections showed that the epidermal chloroplast had higher sensitivity to high CO₂ than the mesophyll chloroplast in *O. cordata* floating leaves, which differs from the responses in *O. alismoides* (Han et al., 2020), where both epidermal and mesophyll chloroplasts showed sensitive responses to CO₂ elevation and the size of both type of chloroplasts were increased. However, it should be noted that, in both *O. cordata* and *O. alismoides*, the area ratio of starch to chloroplast was much greater in mesophyll than in epidermal cells regardless of the CO₂ concentrations.
Responses of CCMs in *O. cordata* Floating Leaves to Variable CO₂

The pH-drift experiment confirmed the results reported by Cao et al. (2019), that floating leaves of *O. cordata* are able to use HCO₃⁻. As a general trend, amphibious heterophyllous plants, in contrast to consistently submerged rooted macrophytes, are not able to use HCO₃⁻ for photosynthesis (Madsen and Sand-Jensen, 1991). However, clearly, *O. cordata* floating leaves exhibited substantial capacity for HCO₃⁻ utilization, irrespective of the CO₂ concentrations during acclimation. Moreover, the HCO₃⁻ utilization efficiency was relatively similar as that in other truly submerged bicarbonate user, such as *O. alismoides* (Zhang et al., 2014; Shao et al., 2017). Considering the much higher concentration of free CO₂ (380–1603 µM) in high CO₂ acclimated conditions, the finding of relatively efficient HCO₃⁻ extraction in *O. cordata* floating leaves grown at high CO₂ was also surprising, because at least some aquatic plants decrease investment in the capacity for HCO₃⁻ utilization when living in an environment with sufficient CO₂ (Maberly and Spence, 1983; Madsen and Sand-Jensen, 1987). From a cost–benefit point of view, under high CO₂ conditions, investment in a CO₂-concentrating system could be expected not to be profitable since the benefit in terms of carbon gain will most likely be restricted (Maberly and Gontero, 2017). However, Madsen and Maberly (1991) reported that the HCO₃⁻ user *Ranunculus peltatus* still benefit significantly from HCO₃⁻ usage even at very high CO₂ concentrations (greater than 200 µM). Overall, the patterns of inorganic carbon depletion in the pH-drift system confirm that the floating leaves of *O. cordata* are a constitutive bicarbonate user.

Based on the enzymatic activity analysis, *O. cordata* floating leaves have the carboxylating, PEP regenerating and decarboxylating enzymes that are needed for operating a C₄ pathway. We firstly compared the activities of Rubisco, PEPC, and PPDK, there are hints of possible C₄ metabolism exist in O.
cordata floating leaves. Under both CO₂ acclimation conditions, the activity of PEPC was greater than that of Rubisco and the ratio of PEPC to Rubisco was about 1.5 and 4 in low and high CO₂ conditions, respectively. The ratio for O. cordata is lower than Hydrilla verticillata, but similar to those reported for O. alismoides, O. acuminata, and Egeria densa, all of which are regarded as C₄ aquatic plants (Casati et al., 2000; Bowes, 2011; Shao et al., 2017). In contrast, the PEPC/Rubisco ratio in terrestrial C₃ plants and aquatic plants that lack a biochemical concentrating mechanism is substantially less than 1 (Zhang et al., 2014). In addition, the activity of PPDK, that regenerates PEP to ensure the supply of substrate for PEPC, was significantly greater at low vs. high CO₂ and equivalent to that of PEPC. Thus, PPDK should be able to support PEPC activity. As the potential decarboxylating enzymes, the NADP-ME activity was not significantly different at low vs. high CO₂ Previous work has shown that H. verticillata (Magnin et al., 1997) and E. densa (Casati et al., 2000) belong to the NADP-ME C₄-subtype. O. alismoides and O. acuminata are the first reports of aquatic plants that belong to the NAD-ME C₄-subtype (Zhang et al., 2014).

C₄ metabolism in some freshwater macrophytes is induced or up-regulated under low/limited CO₂ conditions, such as in H. verticillata and E. densa (Casati et al., 2000; Bowes, 2011). In Eelcharis vivipara, the C₄ cycle is present when its leaves are in air but absent when in water (Murphy et al., 2007). In the present study, it seems that the C₄ cycle is not abolished in high CO₂ acclimated O. cordata floating leaves, which implies that C₄ is constitutive in O. cordata floating leaves, like the other known two constitutive C₄ species in the genus Ottelia: O. alismoides and O. acuminata (Zhang et al., 2014; Shao et al., 2017; Han et al., 2020). C₄ plants require structural and functional coordination to operate C₄ efficiently. For H. verticillata, it performs single-cell C₄ photosynthesis, where C₄ production and decarboxylation occur in different parts of a cell (Bowes et al., 2002). For O. alismoides, a dual-cell C₄ model was assumed, where the epidermal cells produce C₄ product and the mesophyll cells decarboxylate the C₄ product; however, it is also possible that both processes could occur within the mesophyll cells (Han et al., 2020). For O. cordata, further research is needed to establish the precise location of key photosynthetic enzymes involved in the primary CO₂ fixation and decarboxylation, which could help to clarify the specific structural basis for C₄ cycle, as well as to confirm the C₄-subtype in this species.

Aquatic CAM metabolism was firstly found in the freshwater lycophyte Isoetes howellii (Keeley, 1981), and subsequently found in other freshwater angiosperms such as Crassula helmsii (Newman and Raven, 1995), Littorella uniflora (Robe and Griffiths, 2000), O. alismoides (Zhang et al., 2014) and Deinostema violaceum (Yin et al., 2017). In CAM plants, a high nocturnal PEPC activity allows malic acid to be produced in the dark. In O. cordata floating leaves, PEPC remains active at night and its nocturnal activity was very high. Under low CO₂ condition, the PEPC : Rubisco ratio was ~2, which was consistent with nocturnal carboxylation as a consequence of CAM activity. In addition, the diel fluctuations of acidity have been considered to be an important indicator to estimate CAM activity (Keeley, 1998). In the present study, only low CO₂ acclimated O. cordata floating leaves exhibited marked diurnal titratable acidity fluctuation, ~20 µequiv g⁻¹ FW, which was slightly lower than that found in O. alismoides (up to 34 µequiv g⁻¹ FW) (Zhang et al., 2014). It is also lower than Vallisneria americana and V. spiralis, which present evidence for CAM operation with diel acidity changes up to 42 and 51 µequiv g⁻¹ FW, respectively (Keeley, 1998). Nevertheless, similar or lower values have been reported in a number of other putative freshwater CAM species (Webb et al., 1988; Keeley, 1998). In contrast, when grown at high CO₂, O. cordata floating leaves showed a small but not statistically significant diel change in acidity, which indicates the absence of CAM. The field experiments performed by Cao et al. (2019), where the samples of O. cordata floating leaves were collected from their natural stands with the environmental CO₂ concentration ~120 µM, showed the presence of CAM with 12.5 µequiv g⁻¹ FW diel acidity change. This different performance of CAM activity between O. cordata floating leaves grown in situ and in laboratory cultures, might be the consequence of complex environmental variables in the field. CAM activity in aquatic plants, could be affected by a combination of factors including CO₂ concentration, light intensity, temperature, as well as the leaf aging (Maberly and Gontero, 2017; Huang et al., 2018). Thus, the results presented here confirmed that CAM metabolism is present but facultative in O. cordata floating leaves.

Generally, malate is the primary product of CAM photosynthesis and presents substantial diel fluctuations (Keeley, 1998; Igamberdiev and Eprinets, 2016). At night, the final C₄ product malic acid is accumulated and stored in the vacuole; during day, malate comes out of the vacuole and is decarboxylated to produce CO₂ (Borland and Taybi, 2004). In O. cordata floating leaves, we detected five types of organic acids and only malic acid revealed marked diurnal fluctuations at low CO₂, the other four types of organic acid including citric acid, aspartic acid, trans-acconitic acid and fumaric acid were quite stable throughout the day. The significant decrease of malic acid during day suggests that it was most likely decarboxylated to produce CO₂ for photosynthesis in O. cordata floating leaves when grown at low CO₂, as it is in most of the aquatic CAM plants (Keeley, 1998). The nocturnal accumulation level of citric acid is similar with malic acid, however, the role of citrate accumulation in CAM plants is still unclear. Lüttge (1988) reported that CAM plants would transform a part of stored malate to citrate via tricarboxylic acid cycle (TCA cycle) to maintain their metabolic balance. Winter and Smith (1996) reported that citrate may serve to increase the pH-buffering capacity in the vacuoles of CAM plants, which would enhance accumulation of malate. The present data showed that the ratio of nocturnal citric acid accumulation to malic acid accumulation was 33% and 67% for high and low CO₂ acclimated O. cordata floating leaves, respectively. In the CAM plant Euphorbia milii, citrate and malate accumulated equally (Herrera, 2013); whereas, Misalski et al. (2013) found that in the CAM tree Clusia hilariana, malate and citrate experienced independent fluctuations, which were related to photosynthesis and
respiration, respectively. In addition, in O. cordata floating leaves, aspartic acid and trans-aconitic acid presented much higher level than malic acid, but did not respond to CO₂ availability, regardless of dusk and dawn. During metabolism the intensity of converting reactions among different carbon-compounds can cause preferential and higher accumulation of other organic acids than malic acid (Igamberdiev and Eprintsev, 2016). Moreover, aspartic acid content might be partially contributed by its derivative -asparagine, since these two amino acids are not separated quite well on the Athena C18-WP column.

CONCLUSIONS

We conclude that the anatomical structure and CCMs in O. cordata floating leaves exhibits responses to CO₂ availability. High CO₂ acclimation increased the size of upper epidermal cells, but decreased the area of chloroplast in epidermal cells. Under high CO₂, O. cordata floating leaves are able to use HCO₃⁻ in addition to CO₂ as an inorganic carbon source for photosynthesis, and can perform C₄ metabolism. When exposed to low CO₂, it also exhibits CAM cycling characteristics. Whether this species could perform other strategies under different CO₂ conditions, such as the uptake of CO₂ from sediment, is not known yet. Overall, the floating leaves of O. cordata present flexible and efficient ability to adjust structure and CCMs for utilization of inorganic carbon, allowing this species to form dense biomass and to be successfully distributed in shallow waters (He and Sun, 1990). Further studies are needed to establish the detailed biochemical pathway for C₄ and CAM metabolism in this species, as well as the regulatory mechanism among the three CCMs and the exploitation strategies for exploiting atmospheric CO₂, responding to the variable CO₂ availability in the habitat.

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DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

WH and SH designed the experiments. WH, SH, and ZX performed the experiments and collected the data. WH, SH, and WL analyzed and prepared the manuscript. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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