RESEARCH PAPER

In situ O\textsubscript{2} dynamics in submerged Isoetes australis: varied leaf gas permeability influences underwater photosynthesis and internal O\textsubscript{2}

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Received 11 March 2011; Revised 17 May 2011; Accepted 18 May 2011

Abstract

A unique type of vernal pool are those formed on granite outcrops, as the substrate prevents percolation so that water accumulates in depressions when precipitation exceeds evaporation. The O\textsubscript{2} dynamics of small, shallow vernal pools with dense populations of Isoetes australis were studied in situ, and the potential importance of the achlorophyllous leaf bases to underwater net photosynthesis (PN) and radial O\textsubscript{2} loss to sediments is highlighted. O\textsubscript{2} microelectrodes were used in situ to monitor pO\textsubscript{2} in leaves, shallow sediments, and water in four vernal pools. The role of the achlorophyllous leaf bases in gas exchange was evaluated in laboratory studies of underwater PN, loss of tissue water, radial O\textsubscript{2} loss, and light microscopy. Tissue and sediment pO\textsubscript{2} showed large diurnal amplitudes and internal O\textsubscript{2} was more similar to sediment pO\textsubscript{2} than water pO\textsubscript{2}. In early afternoon, sediment pO\textsubscript{2} was often higher than tissue pO\textsubscript{2} and although sediment O\textsubscript{2} declined substantially during the night, it did not become anoxic. The achlorophyllous leaf bases were 34% of the surface area of the shoots, and enhanced by 2.5-fold rates of underwater PN by the green portions, presumably by increasing the surface area for CO\textsubscript{2} entry. In addition, these leaf bases would contribute to loss of O\textsubscript{2} to the surrounding sediments. Numerous species of isoetids, seagrasses, and rosette-forming wetland plants have a large proportion of the leaf buried in sediments and this study indicates that the white achlorophyllous leaf bases may act as an important area of entry for CO\textsubscript{2} or O\textsubscript{2} with the surrounding sediment.

Key words: Aerenchyma, aquatic plant, CAM, malate, photorespiration, sediment O\textsubscript{2}, submergence tolerance, tissue O\textsubscript{2}, underwater photosynthesis, vernal rock pools, wetland plants.

Introduction

Ephemeral wetlands are formed throughout the world during periods where precipitation exceeds evaporation, typically during winter and early spring. Some of these wetlands cycle on an annual basis between periods of standing water and a brief period of waterlogging followed by extreme desiccation, and have been termed ‘vernals pools’ in Mediterranean-type environments (Keeley and Zedler, 1998). A unique type of vernal pool are those formed on granite outcrops, as the granite forms an impervious substrate that prevents downward percolation so that even small depressions in the rock result in standing water. Granite rock pools are 100% precipitation fed, are often very shallow (<10 cm deep), and the substrate of weathered granite and debris blown in from the surrounding land is only a few centimetres thick, resulting in the waterlogged phase being very brief (Keeley and Zedler, 1998; Keeley, 1999). As a consequence, the aquatic flora and fauna of granite rock pools are highly specialized to cope with the extreme environment of these large annual changes. In the present study, in situ tissue O\textsubscript{2} dynamics of completely submerged Isoetes australis, were examined and the role of the achlorophyllous leaf bases for gas exchange with the surrounding sediment evaluated.

During the submergence phase, the plants also need to cope with large diurnal fluctuations in temperature and
dissolved O₂ and CO₂ that occur in these soft-water, shallow pools (Keeley and Zedler, 1998). At night, system respiration processes consume O₂ and produce CO₂ which can lead to hypoxia in the water column (<4 kPa O₂), while CO₂ builds up to concentrations 10- to 15-fold that of atmospheric equilibrium (Keeley and Busch, 1984). During the daytime, when photosynthesis prevails over system respiration, the shallow water column along with a relatively high plant biomass drive pH and O₂ up while CO₂ decreases to <1 μM (calculated from pH and alkalinity; Keeley and Busch, 1984). Underwater photosynthesis of the completely inundated vegetation is thus challenged by high afternoon temperatures and high O₂ in combination with low CO₂, a situation that promotes photorespiration (Pedersen et al., 2011). As a result, carbon-concentrating mechanisms such as C₄ or CAM photosynthesis and bicarbonate use are common in plants inhabiting vernal pools (Keeley, 1999).

Although shallow and transient, vernal pools can host a wide array of aquatic flora and fauna and the Isoetes genus is commonly a major component of the vegetation (Keeley and Zedler, 1998). Species of Isoetes are lycopsids at the same organizational level as ferns and all have hollow quill-like leaves arising from a central corn. Most, if not all, Isoetes species tested so far exhibit indicators of CAM photosynthesis such as dark fixation of CO₂, overnight accumulation of malate followed by daytime decarboxylation, and large diurnal amplitudes in tissue acidity (Keeley, 1998b). However, CAM is often lost in specimens that develop functional stomata upon air exposure (Keeley et al., 1983). The CAM photosynthetic mechanism is of great advantage in the shallow pools as CO₂ can be fixed during the night-time when it is readily available and subsequently released internally and used in daytime photosynthesis at a time when CO₂ becomes depleted in the water (Keeley, 1998a, b, c). As an example, I. australis has recently been shown to possess CAM and in addition to increased underwater photosynthesis at low external CO₂ concentrations, the CAM activity also greatly reduced apparent photorespiration at external O₂ concentrations up to 2.2-fold that of atmospheric equilibrium (Pedersen et al., 2011).

Isoetes species belong to a polyphyletic group of plants of the isoeid life form; these use sediment-derived CO₂ for underwater photosynthesis (Madsen et al., 2002). By using sediment-derived CO₂, these plants tap into an alternative source of CO₂ that is not readily available to plants that do not possess the isoeid growth form (Winkel and Borum, 2009). Isoetids have short, stiff leaves in a basal rosette with large gas-filled lacunae and mostly unbranched porous roots, facilitating diffusion of CO₂ from the surrounding sediment into the roots and up to the leaves. The leaves are often covered with a thick cuticle (Sculthorpe, 1967) reducing the loss of CO₂ to the surrounding water. Consequently, O₂ produced in underwater photosynthesis is not lost to the surrounding water column but diffuses into the roots and out to the rhizosphere (Sand-Jensen and Prah, 1982). As a consequence, the rhizosphere of isoeids is oxidized and free molecular O₂ may even persist throughout a diurnal cycle (Pedersen et al., 1995; Sand-Jensen et al., 2005; Møller and Sand-Jensen, 2011).

The in situ O₂ dynamics of small, shallow vernal pools with dense populations of I. australis were studied; a system contrasting markedly in depth of sediment and water column with those in which submerged plant O₂ dynamics have previously been evaluated. With only a few centimetres of substrate of low organic matter content on top of bedrock, comparison of diurnal O₂ dynamics in these systems with the lake and ocean situations in previous studies (Greve et al., 2003; Borum et al., 2005; Sand-Jensen et al., 2005; Møller and Sand-Jensen, 2011) is of interest. The shallow vernal pools probably dry up and refill a few to several times during spring and so I. australis faces periods of waterlogging or drained conditions with the leaves exposed to air and hence, underwater net photosynthesis (PN), CAM activity, and leaf morphology were compared in both aquatic (produced under water) and aerial (produced in air) leaves. It was observed that the white achlorophyllous leaf bases buried in the shallow sediment accounted for a large proportion of shoot tissue area, with green tips extending only ~15 mm into the shallow water column. This observation prompted testing of the hypothesis that CO₂ entry via these leaf bases might enhance underwater PN, and that these could also be sites of O₂ loss to sediments. It was found that sediment O₂ concentrations never differed significantly from intra-plant O₂ concentrations, indicating easy O₂ exchange between plants and sediment and the basal leaf portions enhanced underwater PN by the green tips, and presumably also contributing substantially to O₂ loss to the sediment.

Materials and methods

Study site and plant materials

Granite outcrops with numerous shallow vernal pools near Mukinbudin, Western Australia (118.2896° E, 30.7468° S) were chosen as the site for all in situ studies and as a collection point for plants used in laboratory and anatomical studies (Fig. 1a). The vernal pools have dense populations of I. australis S. Williams (Fig. 1b) when the pools are water filled from July to October in most years, depending on rainfall (Tuckett et al., 2010). Plants for laboratory work were collected as intact turfs in 5 l buckets and transported back to a 15/20 °C night/day phytotron facility at the University of Western Australia.

In the phytotron, plants were maintained in submerged cultures, 3–5 cm below the surface of ‘artificial floodwater’ (defined below) that was topped up with deionized water to replace evaporation. In one experiment, previously fully submerged plants were de-submerged and maintained in waterlogged sediment, with shoots in air, for 5 weeks. Leaves formed in air were compared with those of plants in a continuously submerged treatment. The artificial floodwater was a solution made in deionized water with final electrical conductivity (EC) of 85 μS cm⁻¹ and containing (in mol m⁻³): 0.15 Ca²⁺, 0.10 Mg²⁺, 0.10 K⁺, 0.30 Cl⁻, 0.10 SO₄²⁻, and 0.10 HCO₃⁻. This low-EC solution was designed to simulate the water in the temporary vernal pools on the granite outcrops from which the specimens were collected. In the phytotron, I. australis continued to grow with a leaf turnover rate of about one leaf per week. Seedlings of the genera Glossostigma and Crassula were removed weekly in order to keep I. australis as a monoculture. Leaf tissues were used for up to 6 weeks following turf collection.
I. australis electrodes were positioned using a magnifying glass and then aluminium stands fixed to a block of concrete, and the micro-Micromanipulators with microelectrodes were mounted on electrodes (OX50-UW; Unisense, Aarhus, Denmark) were used. Unisense, Aarhus, Denmark) and 50 l underwater pA meters with built-in data loggers (PA3000UP-OP; Onset Computer Corporation, Pocasset, MA, USA). Water pH was measured every hour using a handheld pH meter (RH-203; Radiometer, Bronshøj, Denmark). Available CO₂ was calculated using the alkalinity, temperature, and pH data and the formulae of Mackereth et al. (1978). Data were recorded from midday, including throughout the dark period, until the following midday.

**Underwater P₅**

Underwater P₅ by leaves was measured using the method described in Pedersen et al. (2011). Leaf tips (5–10 mm), without the achlorophyllous white base (hereafter referred to as green tips), were excised, the cut end sealed with paraffin, and placed in glass vials (10 ml) with stoppers. Each vial contained two leaf tips in incubation medium, and two glass beads for mixing as these rotated on a wheel within an illuminated water bath (20 °C). PAR inside the submerged glass vials, was 350 μmol m⁻² s⁻¹ (measured using a 4π US-SQS/L; Walz, Effeltrich, Germany). To evaluate the importance of the white achlorophyllous bases of the leaves for gas exchange (CO₂ uptake and O₂ loss), leaves with the white base present and the cut end sealed with paraffin were also incubated in flasks and P₅ rates compared with those of the green tips.

The incubation medium was the same composition as the artificial floodwater (see above) but with 75 μM KHCO₃, 2.5 mol m⁻³ MES with pH adjusted to 6.00 using KOH to achieve 50 μM free CO₂ in the solution. The dissolved O₂ concentration in the incubation medium was set at 50% of air equilibrium, by bubbling with 1:1 volumes of N₂ and air (prior to the addition of KHCO₃); this avoided excess build-up of O₂ during the measurements. As vials were incubated in light immediately after adding the leaves, and since these produce O₂ when in light, there was no risk of tissue hypoxia. Vials without leaves served as blanks.

Following incubations of known duration (60–75 min), dissolved O₂ concentrations in the vials were measured using a Clark-type O₂ microelectrode (Revsbech, 1989; OX-25; Unisense A/S, Aarhus, Denmark), connected to a pH meter (PA2000; Unisense A/S, Aarhus, Denmark). The electrode was calibrated immediately prior to use.

Projected areas of leaf samples were measured using a leaf area meter (LI-3000; LI-COR, Lincoln, NE, USA). Projected areas were converted into actual surface areas using an empirical relationship obtained from measurements of areas based on leaf diameter and length of the cylindrical leaves and conical tips. The ratio of actual area to projected area = 3.633; n=10, see Pedersen et al. (2011) for details.

**ROL from green leaf tips and white achlorophyllous bases**

Radial O₂ loss (ROL) was measured from green and white portions of individual cylindrical leaves to determine resistance to diffusion across cell layers exterior to the lacunae. Leaves were excised and then the tip (3–4 mm) of each leaf was excised, so as to provide a direct path to the lacunae and this end was inserted into a conical
plastic holder connected to a tube (internal diameter 5 mm, length 60 mm) open to the atmosphere. The holder with leaf inserted was mounted using putty (BlueTac; Bostick, Wauwatsa, WI, USA) in a Perspex tank (length 200 mm), containing deoxygenated stagnant 0.1% w/v agar solution also containing: K+, Cl− (5.0 mol m−3) and Ca2+, SO4 2− (0.5 mol m−3). The tube enabled internal O2 to be manipulated, by connecting the tube to a reservoir either of air (21.6% O2) or air enriched with O2 (30% O2). ROL from four replicate leaves was measured at two positions, one each in the green and white achlorophyllous regions, and each at both internal O2 concentrations, using cylindrical O2 electrodes (internal diameter 2.25 mm, height 5.0 mm) fitted with guides to keep each cylindrical leaf near the centre of each electrode (Armstrong and Wright, 1975; Armstrong et al., 1994). Internal O2 concentrations in the green and achlorophyllous regions in four other replicate leaves held in the same set-up were measured using O2 microelectrodes (see above). Diameter of each leaf was measured at the centre of each ROL position, using digital callipers, and the proportion of the outer cell layers (i.e. exterior to the lacunae) to the radius within each tissue zone was determined from hand-cut cross-sections (across all four lacunae, in four replicate leaves). Surface area was calculated assuming that the tissue within the electrode was cylindrical. Resistance to O2 movement across the cell layers external to the lacunae was calculated according to the equations in Garthwaite et al. (2008).

Evaporation from leaf surfaces

Evaporation of water vapour was used as a proxy for cuticle resistance as these surfaces lack stomata, assuming that resistance to water vapour loss might reflect also resistance to movement of other gases (e.g. O2 and CO2). Evaporation from green tips of aquatic leaves (leaves produced under water) and aerial leaves (leaves produced in air; see above) was compared, for tips excised and with the cut surface sealed with paraffin. The experiment was conducted in a desiccator containing desiccant (silica gel) and evaporation was followed over time by weighing groups of five leaf tips initially every 15 min and then every hour. Evaporation rates were calculated as loss in mass over time assuming a density of water of 1 kg l−1. Using the same procedures, evaporation from green aquatic leaf tips was also compared with that from white leaf bases of aquatic leaves. Data were fitted to an exponential loss function (GraphPad Prism 5.0).

Sediment characteristics and sediment O2 consumption

From each vernal pool, three sediment cores (diameter 7.1 cm) were collected in the same area where the in situ O2 measurements took place. Plants were counted to provide a density measurement and shoots, corms, and roots were separated and oven-dried at 70 °C. Another three cores were taken from each site to measure sediment organic matter as ‘loss on ignition’ (5 h at 550 °C).

<table>
<thead>
<tr>
<th>Sediment characteristic</th>
<th>Mean ± SE</th>
<th>SD</th>
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<tbody>
<tr>
<td>Water content (%)</td>
<td>32.0 ± 1.4</td>
<td>7.6</td>
</tr>
<tr>
<td>Organic matter (%)</td>
<td>68.0 ± 2.1</td>
<td>10.4</td>
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Table 1. Proportions, tissue water content, internal leaf lacunal O2 concentration, and rates of radial O2 loss from green tips and white achlorophyllous bases of I. australis leaves

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The potential biological O2 demand (BOD) of the sediment was measured by incubating 1 ml of fresh sediment in 40-ml vials in the dark in artificial floodwater initially at air equilibrium (see underwater PN for chemical composition). Sediment samples were first pre-incubated for 12 h in 5 ml of water bubbled with air to eliminate chemical O2 demand (Raun et al., 2010). The vials were then filled with air-bubbled artificial floodwater, closed, and incubated at 20 °C in the dark on a rotating wheel for 1–1.5 h. O2 concentrations were measured before and after incubation using an O2 minielectrode (OX500; Unisense, Aarhus, Denmark). BOD was calculated as O2 consumption per sediment volume and unit time (nmol O2 l−1 s−1).

Anatomy

Transverse sections were cut, using a hand-held razor, from leaves of field collected plants. Sections were viewed with a light microscope (Zeiss Axioskop2; Carl Zeiss Pty Ltd, Germany), with chloroplast visualization augmented by ultraviolet (UV) light, which causes red chlorophyll autofluorescence. Images were captured with a digital camera (Zeiss AxioCam MRc Rev.3; Carl Zeiss Pty Ltd, Germany) and manipulated for light levels, contrast and brightness using a software package (AxioVision Rel. 4.6; Carl Zeiss Pty Ltd, Germany).

Data analysis

GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA) was used for one- or two-way ANOVA (with Tukey or Bonferroni post hoc test) and Student’s t-test to compare means. If needed, data were log-transformed to improve normality and fulfill requirements of homogeneous variance. GraphPad Prism was also used to fit data sets to predictive models (one-phase exponential decay) in experiments with evaporation.

Results

In situ O2 dynamics and vernal pool characteristics

I. australis grows with a large portion of each leaf (i.e. leaf bases) buried within the sediment, with this buried portion appearing to lack pigmentation (Fig. 1b, c). Leaves of plants collected from vernal pools in M UKinbudin showed pigmentation only in the upper ~71% of their length (Table 1).

Aside from the difference in pigmentation, the green and white leaf portions look superficially similar, being cylindrical—although the tips of the green portion then become conical in shape to form a pointy end. Both segments contain
four large lacunae; however, some aspects of the anatomy of the sections differed between the green and white portions. The green portions contain chlorophyll (red autofluorescence, Fig. 1c) whereas the white bases are achlorophyllous (Fig. 1g) and neither the green tips nor the white bases form stomata. The green portions have a thicker cortical layer (~3.4 ± 0.3 cells; mean ± SE, n=5) compared with the white bases (~2.2 ± 0.3 cells; mean ± SE, n=5), which is also evident from the significantly higher contribution green tips make to the total dry mass. The water contents of the two sections were similar (Table 1). The cuticle was evident on the green tips, but not the white bases (Fig. 1c, g) suggesting a very thin or absent cuticle in these basal portions. This thinner cuticle is presumably more permeable as indicated by the initial 3.6-fold greater rate of loss of water vapour from the white bases as compared with the green tips (Fig. 6a).

**In situ** O₂ dynamics of the leaf lacunae of *I. australis*, the sediment, and the overlying water were obtained from four different vernal pools in close proximity to each other (<25 m) over three consecutive days early in October 2009, springtime in the study region. Measurements using O₂ microelectrodes showed that pO₂ in leaf lacunae and the sediment closely resembled each other both in daylight and during the night (Fig. 2a, Table 2). Figure 2 shows a typical trace of pO₂ in leaf lacunae, sediment, and the overlying water, and in this example O₂ in the lacunae was slightly above that of the surrounding sediment on the first day with maximum values of 30 kPa. In the late afternoon, pO₂ in leaf lacunae and sediment declined rapidly, continuing to decline throughout the night, reaching values as low as 1.6 kPa by dawn (Table 2). The overlying water column also declined overnight but to a lesser extent than the leaf lacunae and sediments. Upon sunrise the next day, pO₂ increased in both leaf lacunae and sediment to values similar to those of the day before, although pO₂ in the sediment on day 2 was higher than in the leaf lacunae. This pattern was consistent in all four replicates and minimum or maximum intra-plant pO₂ never significantly differed from the surrounding sediment either during the day or during the night (Table 2). The water column always had higher minimum night-time pO₂ than the sediment and the plant tissues, but lower maximum values during the day (Table 2). Although the pO₂ in both sediment and leaf lacunae declined throughout the night to ~3 kPa in three replicates, however, in two replicates, leaf lacunae became severely hypoxic with pO₂ down to 0.3 kPa (Supplementary Fig. S1 at *JXB* online).

Day 1 in the example shown in Fig. 2 was a cloudy and windy day and water column temperature fluctuated by ~20 °C. During the following calm and clear night, water temperature dropped to 5.5 °C and iced formed on top of some of the shallowest vernal pools without plants. Only 6 h after the minimum water temperature was recorded, a maximum of 30 °C was recorded the next day (sunny) in the same pool (Fig. 2b).

Plant density of *I. australis* was relatively uniform within vernal pools, but varied by >20-fold among vernal pools (250–5400 plants m⁻²; Fig. 3a). The variation in density among pools was also reflected in differences in biomass (6–64 g DM m⁻²; Fig. 3b) and hence, individual plant mass was not affected by plant density (Fig. 3c). Allocation into root biomass, corm, and shoot was similar for plants among vernal pools (Fig. 3d), potentially explaining why *in situ* O₂ dynamics were remarkably similar in all four pools tested (Supplementary Fig. 1S available at *JXB* online).

Sediment organic matter was measured as there is a strong, often linear, relationship between sediment organic matter and sediment O₂ consumption (Raun *et al.*, 2019).
Sediment organic matter varied little between the four vernal pools where the \textit{in situ} O$_2$ dynamics were measured (vernal pool Nos 1–4; Fig. 3e), but the sediment O$_2$ consumption was variable among vernal pools and also within each pool (Fig. 3f).

**Underwater P$_N$ and O$_2$ exchange via the achlorophyllous leaf bases**

Isoetids, including species of \textit{Isoetes}, are known to lose a considerable share of the O$_2$ produced in underwater P$_N$ via the roots as the leaves are often less permeable to gases than the roots (Sand-Jensen and Prahl, 1982). It was noticed that most specimens had a substantial proportion of the lower portions of leaves buried in the sediment and the buried leaf bases did not contain chloroplasts and had a less obvious cuticle (Fig. 1b, c, f, g). Thus, it was hypothesized that the white bases of leaves could be areas of entry for CO$_2$ and thus enhance underwater P$_N$, as well as contribute to radial O$_2$ loss to the surrounding sediments.

The achlorophyllous leaf bases contained very little malate [\(<2\ \mu$mol g$^{-1}$ fresh mass (FM)] and citrate [\(<0.5\ \mu$mol g$^{-1}$ FM] and neither varied significantly over the diurnal cycle. In contrast, the diurnal amplitude of malate in green leaf tips was 82 \(\mu$mol g$^{-1}$ FM while that of citrate was 7 \(\mu$mol g$^{-1}$ FM (Fig. 4a, b). Thus, the white bases do not contribute to night-time storage of organic acids that can be decarboxylated during the subsequent day.

It seems, however, that the white bases could act as entry point for CO$_2$, exit point for O$_2$, or both. Whole excised leaves with the white bases present and sealed at the cut surface with paraffin had a 2.5 higher underwater P$_N$ compared with green leaf tips where the white base had been removed (cut surface also sealed), demonstrating that CO$_2$ entry via the white bases can greatly enhance underwater P$_N$ (Fig. 5). This effect of enhanced CO$_2$ entry with white bases intact probably resulted from the 1.5-fold larger surface area with these present, compared with the green tissues only.

Measurements of ROL from the white base and green areas of leaves, when in darkness in an O$_2$-free medium but with O$_2$ supplied to the lacunae via a tube connected to air (20.6%) and then O$_2$-enriched air (30%), provided rates of ROL as well as enabling calculation of the resistance to O$_2$ loss across the external cell layers within the two zones. Although internal O$_2$ concentrations were significantly lower in the lacunae of the white tissues (Table 1), the actual ROL rates did not differ significantly (442 nmol m$^{-2}$ s$^{-1}$ for green tips and 527 nmol m$^{-2}$ s$^{-1}$ for white bases). The rates show

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**Fig. 3.** Variation in plant density (a), individual plant mass (b), biomass allocation to shoot, corm, and root (c), biomass (d), sediment organic matter (e), and sediment O$_2$ consumption (f) in four granite vernal pools inhabited by \textit{I. australis}. Means $\pm$ SE, \(n=4\).
potential for ROL from white portions when an O$_2$ gradient exists to the sediment. The mean resistance of the external cell layers to radial O$_2$ diffusion did not differ between the two zones, and was $3.08 \pm 10^5$ sc m$^{-3}$.

Difference in resistance to O$_2$ loss within the two tissue areas were expected, as white bases initially lost water vapor 3.6-fold more compared with green tips (cut ends sealed in both cases, Fig. 6a) and microscopy of leaf cross-sections revealed a prominent cuticle on the green tips but this feature was not apparent on the white bases (Fig. 1e, g); so the cuticle is presumably not the major component of resistance to ROL from the lacunae within these shoots.

In summary, measurements of underwater P$_N$, radial O$_2$ loss, evaporation from tissue segments exposed to dry air, and microscopy, all support the hypothesis that the white leaf bases may enhance the surface area available for entry of CO$_2$ and exit of O$_2$ (both depending upon diffusion gradients with the external medium), in *I. australis*.

**Do leaves acclimate to air exposure when the water level recedes?**

In its natural habitat, *I. australis* probably faces highly fluctuating water regimes throughout the growth season. The brown tips (Fig. 1b) probably indicate that these leaves had been air exposed at some point during their life, so it was decided to investigate whether air-acclimated leaves are formed when shoots are in air during times with only sediment waterlogging. Aerial leaves, i.e. leaves formed in
air, did show slower evaporation rates compared with aquatic leaves (leaves formed under water; Fig. 6b) indicating that the cuticle is somewhat modified when leaves are formed in air. However, underwater $P_N$ was similar in aerial and aquatic leaves, stomata never formed in aerial leaves, and the CAM activity was also maintained in aerial leaves (Pedersen et al., 2011).

**Discussion**

The present study evaluated diurnal *in situ* $O_2$ dynamics in completely submerged *I. australis*, in the sediment in the shallow vernal pools in which the plants were growing, and in the overlying water column. It was found that sediment $O_2$ concentrations were closely related to intra-plant $O_2$ fluctuations and the resulting diurnal amplitude was 3-fold higher in the sediment than in the water column. In the following, the implications of these findings and possible role of the achlorophyllous basal leaf portions in $O_2$ and $CO_2$ exchange with the surrounding sediment are discussed.

There are few studies of *in situ* $O_2$ dynamics in completely submerged wetland plants. Those of seagrasses all show that intra-plant $O_2$ during the day is a function of light, whereas during the night $O_2$ diffuses into leaves from the surrounding water column, and then into rhizomes and roots as the bulk sediment is permanently anoxic (Greve et al., 2003; Borum et al., 2005; Holmer et al., 2009). Borum et al. (2006) also showed that the dependence on water column for night-time $O_2$ supply is critical; if the water column becomes hypoxic during the night, the risk of tissue anoxia is high. There are only two studies of *in situ* $O_2$ dynamics in an isoetid (*Lobelia dortmanna*). A winter investigation showed that the rhizosphere remained oxic during the night and at one point was even slightly supersaturated during the day (Sand-Jensen et al., 2005). As these measurements were taken during winter, the low temperature (~2 °C) probably resulted in very low microbial respiration and so low sediment $O_2$ consumption. In contrast, a more recent study by Møller and Sand-Jensen (2011) showed that the same *L. dortmanna* population experiences anoxic conditions in the sediment during the summer with much higher temperatures (~20 °C).

The present study on *I. australis* from shallow, vernal pools differs in one crucial aspect from previous *in situ* studies of $O_2$ dynamics. In the vernal pools the sediment is restricted by the underlying biologically inert bedrock; consequently, these pools have a finite volume of sediment with a finite $O_2$ consumption. This situation contrasts with lakes and oceans where the sediment represents an infinite volume, and thus also $O_2$ consumption, ultimately leading to anoxia at certain distances into the sediment (i.e from the $O_2$ source). In vernal pools with a dense population of *I. australis*, $O_2$ builds up to >30 kPa during the day and even with the combined $O_2$ uptake of respiration by plant roots and sediment microbes sediment anoxia did not occur during the night-time in regions near plants (Fig. 2, Table 2). In contrast, bare sediments remained anoxic both in light and dark, further emphasizing the importance of plant-derived inputs of $O_2$ to enable sediments to remain oxic. Interestingly, shortly after sunset, the $O_2$ gradient changes so that the leaves possibly take up $O_2$ from the water column, which subsequently diffuses through the lacunae in leaves and roots and via ROL as a constant source of $O_2$ to the sediment throughout the night. The oxic sediment conditions are not caused by an extremely low sediment $O_2$ consumption per volume of sediment. In fact, the sediment $O_2$ consumption rates in this study (70–130 nmol $O_2$ l$^{-1}$ s$^{-1}$, Fig. 3) were similar to those reported by Raun et al. (2010), who found 114–173 nmol $O_2$ l$^{-1}$ s$^{-1}$ with 0.9–1.7% organic matter, and considerably higher than those from the oligotrophic lake Skånes Värsjö with only 26 nmol $O_2$ l$^{-1}$ s$^{-1}$ (Claus Møller, unpublished data). Hence, the oxic sediment must be caused by the fact that the $O_2$ consumption is limited by the finite sediment volume, which in turn is likely to be approximately equal to the entire rhizosphere from which $O_2$ is leaking during the day in pools with dense *I. australis*.

Interestingly, sediment $pO_2$ at some points during the day exceeded that of intra-plant $pO_2$, a phenomenon observed in all four replicate pools (Fig. 1a, Supplementary Fig. S1 at *JXB* online). This could be due to (i) $O_2$ produced by microalgae on the sediment surface, (ii) $O_2$ produced by neighbouring plants with higher rates of underwater $P_N$ and thus higher ROL, or (iii) the experimental circumstances. Natural sediments normally host a population of benthic microalgae and their photosynthesis can lead to supersaturation of $O_2$ of up to 90 kPa in dense nutrient-rich biofilms (Lassen et al., 1997) but also up to 35 kPa on very nutrient-poor sediments with *L. dortmanna* (Pedersen et al., 1995; Møller and Sand-Jensen, 2011). Vertical $O_2$ profiles of the sediments in the vernal pools were not measured. However, benthic algae were observed in most vernal pools in the granite outcrops and their $O_2$ production would contribute to the supersaturation of the sediment (Lassen et al., 1997) and could, therefore, also result in sediment $pO_2$ exceeding that of the lacunae inside *I. australis*.

Secondly, the high external $pO_2$ in the surrounding sediment could also be caused by high radial $O_2$ loss from neighbouring plant roots. If the neighbouring plants had higher rates of photosynthesis, it would also lead to higher internal $pO_2$ and ultimately to higher ROL and high local $O_2$ concentration in the rhizosphere of some plants. Thirdly, in order to insert the microelectrodes into the small leaves, individuals might have been selected from patches with lower plant density than those nearby from where the sediment measurements were taken. It is not possible to exclude either of the three explanations and a combination of the three seems likely to have caused the high $pO_2$ observed in the sediments containing *I. australis*.

The diurnal amplitudes in $pO_2$ of the overlying water column were significantly smaller than in the sediments (Table 2) but resembled those observed for Californian vernal pools (Keeley and Busch, 1984). The water column of the Californian vernal pools, however, became lower in night-time $pO_2$ presumably as a consequence of higher plant
biodiversity in those pools, which are probably not quite as oligotrophic as the vernal pools of Western Australia that are formed on the granite outcrops. Diurnal CO₂ fluctuations in the present study were, however, in the range reported from Californian vernal pools (Keeley and Busch, 1984) with very low values in the late afternoon (3 μmol l⁻¹; air equilibrium is ~15 μmol l⁻¹) and high concentrations following night-time system respiration (270 μmol l⁻¹, caption of Fig. 2). Water column temperature rose 20 °C in <6 h and the combination of high temperature, high pO₂, and low CO₂ would potentially increase photosynthesis (Pedersen et al., 2011).

Most, if not all, species of Isoetes would often have a large proportion of the leaf tissue buried in the sediment, resulting in white achorophyllous leaf bases. An extreme example is that of Isoetes andicola, a bog plant from the Andes, where only 4% of the total plant mass is allocated to green leaf tips whereas 11% of the total plant mass is in white achorophyllous leaf bases (Keeley et al., 1994). In the present study of I. australis, the cuticle was less obvious in the white achorophyllous leaf bases and these tissues were also more permeable to water vapour loss. However, the resistance of the outer cell layers to radial O₂ diffusion were also more permeable to water vapour loss. However, in the white achorophyllous leaf bases and these tissues I. australis white achorophyllous leaf bases (Keeley et al., 2011).

References


Supplementary data

Supplementary Fig. S1 shows in situ O₂ dynamics in a leaf of I. australis, the sediment of the rhizosphere and the water column and incident light and temperature (days 1–2) over a diurnal cycle in three granite vernal pools.

Acknowledgements

Dion Nichol’s help to obtain permission to survey and work with vernal pools on Allan and Craig Nichol’s property near Mukiinbudin is greatly appreciated. The authors thank Dr Gregory Robert Cawthra for help with HPLC analyses of organic acids and Prof. William Armstrong for help with calculations of resistances to O₂ diffusion derived from our ROL measurements. The financial support of CLEAR, a Villum Kann Rasmussen Centre of Excellence and the Danish Science Council is also acknowledged.


