Turnitin-An improved method for the simultaneous determination of photosynthetic O2

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Submission date: 08-Jun-2023 07:06PM (UTC+0700)
Submission ID: 2111722543
File name: od_for_the_simultaneous_determination_of_photosynthetic-2016.pdf (261.39K)
Word count: 4121
Character count: 22656

DOI: 10.1007/s11099-015-0166-6 PHOTOSYNTHETICA 54 (1): 152-157, 2016

BRIEF COMMUNICATION

An improved method for the simultaneous determination of photosynthetic O_2 evolution and CO_2 consumption in *Rhizophora mucronata* leaves

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Abstract

The photosynthetic gas-exchange has been assessed traditionally either as O_2 evolution or CO_2 consumption. In this study, we used a liquid-phase O_2 electrode combined with CO_2 optodes to examine simultaneously photosynthesis in intact leaves of mangrove *Rhizophora mucronata*. We verified suitable conditions for leaf photosynthetic rates by assessing pH levels and $NaHCO_3$ concentrations and compared these to the gas-exchange method at various PAR levels. The photosynthetic rate in response to pH exhibited a similar pattern both for O_2 evolution and CO_2 consumption, and higher rates were associated with intermediate pH compared with low and high pH values. The net photosynthetic quotient (PQ) of *R. mucronata* leaves ranged from 1.04–1.28. The PQ values, which were never lesser than 1, suggested that photorespiration did not occur in *R. mucronata* leaves under aqueous conditions. The similar maximum photosynthetic rates suggested that all measurements had a high capacity to adjust the photosynthetic apparatus under a light saturation condition. The simultaneous measurements of O_2 evolution and CO_2 consumption using the Clark oxygen electrode polarographic sensor with the CO_2 optode sensor provided a simple, stable, and precise measurement of PQ under aqueous and saturated light conditions.

Additional key words: carbon dioxide consumption; oxygen evolution; photosynthetic performance.

Leaf O_2 evolution and CO_2 consumption are fundamental mechanisms that support oxygen and carbon ecosystems from the individual plant to the global scale. Based on the photosynthesis chemical formula, which justifies that the ratio of O_2 evolution to CO_2 fixation is 1:1 (Espie 1986), the traditional estimation of photosynthetic gas-exchange has been evaluated either by O_2 evolution or CO_2 consumption. However, in an intact leaf, some physiological functions that synthesise and consume O_2 and CO_2 may vary, particularly under stress conditions (Wu *et al.* 2014), photorespiration (Rosenberg *et al.* 1995), and other oxygenative functions (Taddei *et al.* 2008). This means that the ratio of O_2:CO_2 during photosynthesis in intact leaves is not always 1:1.

The simultaneous estimation of O_2 and CO_2 has been done using isotope-gas chromatography-mass spectrometry (GC-MS) with $^{13}CO_2$ and $^{18}O_2$ (Isobe *et al.* 2011). However, the method is unpopular because the equipment is very expensive (Sipior *et al.* 1996). In this study, we tried to improve the potential for a convenient evaluation of O_2 evolution and CO_2 consumption in photosynthesis by using the O_2 electrode and CO_2 optodes simultaneously. The main advantages of optodes are that they can be used in non-invasive systems, oxygen and carbon dioxide are not consumed by the optodes, measurements are possible over a wide temperature range, and there is no mechanical stress (Warkentin *et al.* 2007). If this simultaneous method is convenient, it becomes a useful mechanism to study more easily physiological effects of photosynthesis.

A simultaneous measurement of O_2 evolution and CO_2 consumption during photosynthesis is also essential in order to calculate the photosynthetic quotient (PQ), which is described as the molar ratio of the rate of O_2 production to the rate of CO_2 utilization (Williams and Robertson

Received 8 April 2015, *accepted* 22 June 2015, *published as online-first* 5 August 2015.
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Abbreviations: P_{max} – light-saturated photosynthetic rate; P_N – net photosynthetic rate; PQ – photosynthetic quotient; RuBP – ribulose-1,5-bisphosphate.
Acknowledgements: We acknowledge The United Graduate School of Agricultural Sciences, Kagoshima University, Japan for continuous funding through The Rendai Research Subsidies Program. We also thank Yutaro Oba for his technical support.

1991). Some ecosystem productivity studies have been made with the assumption that PQ = 1 (Suzumura *et al*. 2002, Nielsen and Nielsen 2006); it could affect data interpretation of tropical productivity (Taddei *et al*. 2008).

Mangroves represent an important coastal ecosystem in tropical areas. During the seedling stage, red mangrove (*Rhizophora mucronata* L.) lives periodically in submerged conditions similar to seaweed or macroalgae. Our previous work (Ulqodry *et al*. 2014) explored the photosynthetic performance of *R. mucronata* leaves using the gas-exchange method. This method showed a high precision and was rapid (Moore *et al*. 1973, Sobrado 2005, Okimoto *et al*. 2007), but it was limited under aqueous conditions as the infra-red gas analyser was sensitive to water immersion (Gevaert *et al*. 2011). The advent of a new type of optical electrodes, the so-called opt(r)odes, facilitated the estimation of the *R. mucronata* photosynthetic rate under aqueous conditions. Previous studies have applied optodes for oxygen and carbon independently in the water column, sediments, and plant tissues (Gansert *et al*. 2001, Glud *et al*. 2005, Berggren *et al*. 2012).

In this study, we examined the photosynthetic O_2 evolution and CO_2 consumption rates of *R. mucronata* leaves under aqueous condition. In a simultaneous experiment, we used a liquid-phase O_2 electrode and CO_2 optodes in order to demonstrate their interdependence and differences and compared the results with those of the gas-exchange method. The determination of PQ values and light-saturated photosynthetic rate (P_{max}) of *R. mucronata* was investigated under aqueous conditions.

Propagules of *R. mucronata* were obtained from a mangrove area on the Galang Island, Batam District, Indonesia (0°45'N, 104°15'E). Propagules were initially grown in a heated greenhouse at the Laboratory of Tropical Crop Improvement, Saga University, Japan (33°14'N, 130°17'E). The fully expanded leaves from 3–4 mangrove seedlings were used as materials.

The leaves were collected in early morning, vacuum-infiltrated with the buffer, and stored in the dark until required. One essential consequence of this treatment was the inactivation of Rubisco, so that the photosynthetic rates were approximately 10% of those generally observed from leaves taken directly from a plant (Brown 1998). The leaf sample was sliced into squares of approximately 1 mm^2. The leaves were sliced under a 50 mM HEPES buffer containing 0.5 mM $CaSO_4$ and transferred into the electrode chamber that contained the same buffer.

Photosynthetic O_2 evolution and CO_2 consumption were measured simultaneously in a closed chamber using an aqueous-phase of a Clark oxygen electrode type polarographic sensor (*Hansatech*, Norfolk, UK) with a '*pCO_2 mini'* optodes sensor (*PreSens GmbH*, Regensburg, Germany) that was inserted into the chamber. The optodes system guarantees a high temporal resolution and a measurement without drift, oxygen consumption, or gas-exchange between the incubation chamber and the environment (Warkentin *et al*. 2007). The chamber was equipped with a water jacket to maintain temperature at 25°C. Periodic checking ensured that the highest illumination intensity did not result in a rapid increase in temperature. Light was provided by a slide projector lamp and the lens system focused the light into the electrode compartment. The photosynthetically active radiation in the chamber was measured with a quantum sensor (model *QRT1*, *Hansatech*, Norfolk,UK). It was important that the slices did not obstruct the rotation of the magnetic flea and also the sensor of *pCO_2 mini*. To achieve maximum accuracy, a two point calibration of the sensor and buffer was equilibrated with saturation air 21% and also zero oxygen line by using nitrogen bubble. This process also removed any dissolved CO_2 from the medium, such that the added $NaHCO_3$ was the only carbon source available.

Photosynthetic O_2 evolution and CO_2 consumption of *R. mucronata* leaves under aqueous conditions were measured at various pH, $NaHCO_3$ concentrations, and PAR levels at temperature of 25°C. The relationship between the pH of the buffer and apparent photosynthetic rate was measured at pH 6.0, 6.5, 7.0, 7.5, 8.0, and 9.0 with 20 mM $NaHCO_3$ as carbon dioxide source under saturation PAR of 1,000 µmol(photon) m^{-2} s^{-1}. The effect of different $NaHCO_3$ concentrations (0, 5, 10, 20, and 40 mM) was measured at pH 7.5 and a saturation PAR of 1,000 µmol(photon) m^{-2} s^{-1}. In relation to light intensity, PAR values in the chamber were maintained in decreasing levels from 1,000 to 50 µmol(photon) m^{-2} s^{-1} by placing various distance between the projector lamp and the chamber. For a dark respiration measurement, the electrode chamber was wrapped in two layers of aluminium foil.

The O_2 electrode signal was recorded using *Oxygraph Plus System* software (*Hansatech*, Norfolk, UK) as a real-time chart recorder simulation. Simultaneously, the CO_2 consumption was measured in the same chamber every 5 s using *pCO_2 View v1.0.2* software (*PreSens GmbH*, Regensburg, Germany). There was a lag period lesser than 2 min for CO_2 consumption, and about 3 min for O_2 evolution after light activation (Fig. 1S, *supplement available online*). Generally, the lag period of O_2 evolution was slightly longer than that of CO_2 consumption, but insignificantly. Furthermore, the O_2 evolution and CO_2 consumption rates were calculated from the initial slopes of the curves during a linear photosynthetic activity after the lag period finished.

As a comparison, the photosynthetic rate based on gas-exchange in the air was also performed on the pair of leaves similar to those used to measure O_2 evolution and CO_2 consumption under aqueous conditions. Measurements of leaf gas-exchange were conducted using a portable open-flow gas-exchange system (*LI-6400*, *Li-COR*, Lincoln, NE, USA). The effect of light intensity on the photosynthetic rate was measured from PAR 1,000 to 0 µmol(photon) m^{-2} s^{-1} (1,000; 500, 250, 100, 50, 0 µmol m^{-2} s^{-1}) with leaf temperature, vapour pressure deficit between the leaf and air (VpdL) and CO_2 input of

25°C, 1.7 ± 0.3 kPa, and 370 µmol mol^{-1}, respectively. The light responses of the photosynthetic rate was determined using the rectangular hyperbola model (Okimoto *et al.* 2008) to specify the P_{max} of *R. mucronata* leaves (Ulqodry *et al.* 2014) in air and under aqueous conditions.

Analysis of variance (*ANOVA*) was performed using *StatistiXL Version 1.x.* Significant differences between treatments were further evaluated using the *Tukey*'s HSD test (P<0.05).

We began the experiment by comparing the most suitable leaf shape that would result in the highest O_2 evolution and CO_2 consumption, *i.e.*, small slice pieces (1 mm^2) and a larger, chip shape ones (1 cm^2). Previous results indicated that cutting leaves into small pieces can be negligible during O_2 evolution measurement under aqueous condition (Kawamitsu and Boyer 1999). Our results showed that the small *R. mucronata* leaf sample exhibited significantly higher O_2 evolution and CO_2 consumption rates compared to the larger, chip shape pieces (Fig. 1*A*). This suggests that slicing the leaf tissues facilitated increasing gas-exchange across the boundary layer at the tissue surface (Brown 1998). This eliminates the effect of stomatal resistance for CO_2 diffusion, and free CO_2 molecules or HCO_3^- ions may penetrate more easily into the tissue of the leaf slice, resulting in a higher photosynthetic rate (Ishii *et al.* 1977).

The most important factors for measuring the net photosynthetic rate (P_N) in aqueous conditions were pH and a carbonate system of the reaction mixture. Dissolved carbon dioxide in water occurs in three inorganic forms: free aqueous carbon dioxide (free CO_2), bicarbonate (HCO_3^-), and carbonate ions (CO_3^{2-}). If the equilibrium is affected by a change in pH, this could potentially influence P_N (Riebesell *et al.* 2007). The P_N in response to pH exhibited a similar pattern for both O_2 evolution and CO_2 consumption, with higher rates associated with intermediate pH values of 7.0–7.5 compared to low and high pH (Fig. 1*B*). Under high pH conditions of 8.0–9.0, free molecular CO_2 decreased and bicarbonate increased (Chen and Durbin 1994). This meant that free CO_2 in the reaction mixture became limiting and it reduced P_N. This result also demonstrated that the main carbon form utilised as the substrate for *R. mucronata* leaf photosynthesis was free CO_2 molecules rather than bicarbonate. Almost all terrestrial plants use only free CO_2 for photosynthesis, however, many seaweeds or macroalgae use both free CO_2 and external bicarbonate in water as the source of carbon for photosynthesis (Kawamitsu and Boyer 1999, Pierini and Thomaz 2004). However, the photosynthetic rate, which was detected even at pH 9, indicated that *R. mucronata* leaves used bicarbonate as an additional source of carbon under low free CO_2 conditions. The enriched membrane fragments from oxygenic photosynthesisers (Shevela *et al.* 2012). Bicarbonate is

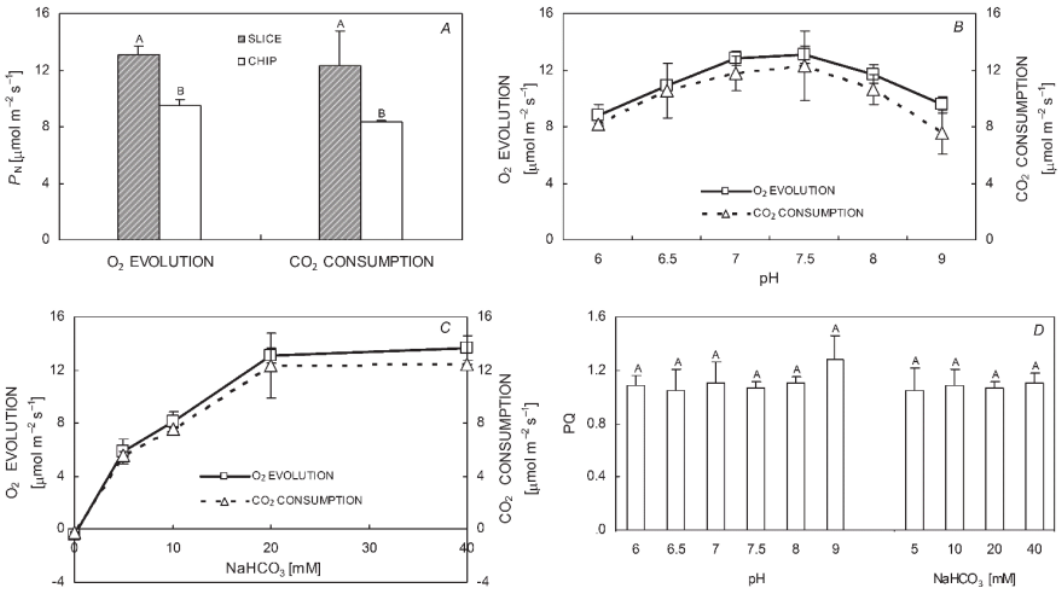

Fig. 1. Photosynthetic O_2 evolution and CO_2 consumption of *R. mucronata* leaves under aqueous conditions measured in various: sample forms (*A*), pH levels (*B*), and $NaHCO_3$ concentrations (*C*); and the photosynthetic quotient (PQ) values as ratio of O_2 evolution and CO_2 consumption (*D*). The conditions of the measurements: temperature of 25°C and PAR of 1,000 µmol(photon) m^{-2} s^{-1}. Values are means ± SD. *Different letters* over bars represent significant differences among all treatments (P<0.05, *Tukey*'s HSD test).

requirement of PSII for bicarbonate (carbonate) has been observed for intact leaves, isolated thylakoids, and PSII-required for the regulation of photosynthetic electron transport on the acceptor side of PSII (Wydrzynski and Govindjee 1975), and is probably also involved in the mechanism of O_2 evolution on the oxidising side of PSII (Stemler 2002).

This study was unable to demonstrate that high free CO_2 under low pH condition (<7.0) resulted in high P_N. It seemed that leaf O_2 evolution and CO_2 consumption were strongly related to leaf intracellular conditions. Berge *et al*. (2010) pointed out that as pH dropped, the H^+ concentration increased which may affect intracellular pH, membrane potential, energy partitioning, and enzyme activity. For this reason, aqueous acidification may reduce P_N through direct pH effects.

Variation in P_N responses to $NaHCO_3$ concentrations also showed almost similar trends for both O_2 evolution and CO_2 consumption. P_N increased with higher $NaHCO_3$ concentrations until reaching the saturation point at 20 mM (Fig. 1*C*). The high bicarbonat saturation point indicated that our method needed very rich carbon dioxide source. Particularly, in submerged plants, P_N may be limited by a low availability of dissolved inorganic carbon (Maberly and Spence 1983, Adamec 1997). The interesting finding was that although there was no significant difference between O_2 evolution and CO_2 consumption, the O_2 evolution values were always higher than those of the CO_2 consumption values under the different pH and $NaHCO_3$ concentrations. This result is important if we want to explore the PQ of *R. mucronata* leaves under aqueous conditions. To be useful, PQ should be determined using the net rate of O_2 involved per CO_2 fixed simultaneously and can be described as:

$$\mathrm{PQ} = \frac{\mathrm{O_2\ evolution}}{\mathrm{CO_2\ consumption}}$$

Stoichiometrically, the PQ value equal to 1.00 assumes a hexose production with ammonium as the N source (Rosenberg *et al.* 1995). If this simple photosynthesis physiology was replaced by an ecological summation of protoplasm production, including carbohydrates, proteins, lipids, and nucleic acids, then the theoretical PQ would be higher (Williams and Robertson 1991). Theoretical PQ values typically range from 1.0 to 1.3 (Rosenberg *et al*. 1995). The PQ values of *R. mucronata* leaves under the different pH and $NaHCO_3$ concentrations ranged from 1.04–1.28 with no significant difference among them (Fig. 1*D*). Purely based on stoichiometric and theoretical considerations of the PQ values, results similar or higher than 1.0 would be expected. PQ of 1.0 infers that the sole product of photosynthesis is carbohydrate, while PQ>1.0 indicates that more reduced compounds are produced, such as fats and proteins (Chisholm 1998). Our results also suggested that the simultaneous measurement of O_2 evolution and CO_2 consumption by using the polarographic sensor of Clark oxygen electrode and the '*pCO_2 mini*' optode sensor provided simple, stable, and precise measurements of net PQ under aqueous conditions.

The net PQ values in all measurements were never lesser than 1.0; it confirmed that photorespiration did not occur under aqueous conditions. A possible explanation for a PQ lesser than 1.0 would be photorespiration (glycolate production) as a result of oxygenase activity of Rubisco at high ambient oxygen concentrations (Rosenberg *et al*. 1995). Photorespiration that decreases PQ occurs when Rubisco, which principally functions as carboxylase, is substituted by its oxygenase function (Taddei *et al*. 2008). In terrestrial C_3 plants, photorespiratory consumption of O_2 can account for 25% of Rubisco activity (Falkowski and Raven 1997). Conversely, photorespiration is assumed to be of minor importance to aquatic plants compared with terrestrial C_3 plants (Laws *et al*. 2000), because submerged environmental conditions, such as fairly constant oxygen and total inorganic carbon concentrations, does not favour photorespiration (Rosenberg *et al*. 1995).

In order to characterise functioning of photosynthetic apparatus of *R. mucronata* in air and under aqueous conditions, the light curves of P_N for similarly paired leaves were estimated. In Fig. 2, at low light levels [PAR < 500 μmol(photon) m^{-2} s^{-1}], the photosynthetic rate of O_2 evolution and CO_2 consumption under aqueous conditions were lower than the photosynthetic CO_2 exchange in air. This result is likely to be related to the reduction of low light utilisation while the leaf slices were rotated under aqueous conditions. Another possible explanation was that our method worked well under light saturation compared to light limitation. Therefore, we needed to improve the simultaneous measurements of photosynthetic O_2 evolution and CO_2 consumption under aqueous conditions under low light.

The light saturation points for all P_N measurements (CO_2 exchange in air, O_2 evolution under aqueous condition, and CO_2 consumption under aqueous condition) were similar at PAR levels around 500–1,000 μmol(photon) m^{-2} s^{-1}. The P_{max}, which demonstrates the potential photosynthetic capacity of *R. mucronata* leaves (Ulqodry *et al*. 2014), was also determined. All experiments produced comparable results with similar P_{max} values of 13.37, 13.11, and 12.31 μmol m^{-2} s^{-1} for CO_2 exchange in air, O_2 evolution under aqueous conditions, and CO_2 consumption under aqueous conditions, respectively. In comparation with gas-exchange, the maximum photosynthetic rate in photosynthetic O_2 evolution and CO_2 consumption under aqueous condition

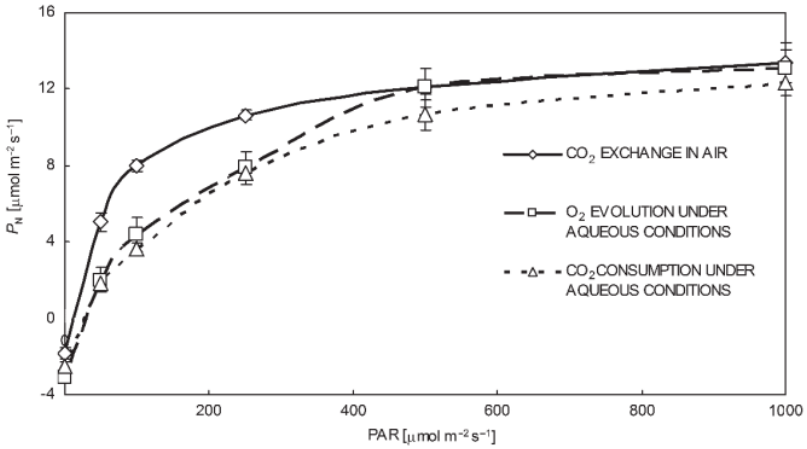

Fig. 2. Response of net photosynthetic rate (P_N) to increasing PAR in the *R. mucronata* leaves. Measurements in air were made with a portable open-flow gas-exchange system, *LI-6400* (CO_2 exchange in air) and measurements under aqueous conditions were made simultaneously with an aqueous-phase O_2 electrode (O_2 evolution) and '*pCO_2 mini*' optode system (CO_2 consumption). Temperature was 25°C for all measurements. Values are means ± SD.

was achieved under very high carbon dioxide concentration. The P_{max} value and daily period of irradiance, when plants were in the water and air, would be useful as an indicator of primary production (Zimmerman *et al*. 1994). The similar P_{max} values suggested that all treatments resulted in a high capacity to adjust the photosynthetic apparatus under light saturation conditions.

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