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ORIGINAL RESEARCH PAPER

Characterization of photosynthetically active duckweed (*Wolffia australiana*) in vitro culture by Respiration Activity Monitoring System (RAMOS)

Henrik Rechmann · Andrea Friedrich · Dara Forouzan · Stefan Barth · Heide Schnabl · Manfred Biselli · Robert Boehm

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Abstract The feasibility of oxygen transfer rate (OTR) measurement to non-destructively monitor plant propagation and vitality of photosynthetically active plant in vitro culture of duckweed (*Wolffia australiana*, Lemnaceae) was tested using Respiration Activity Monitoring System (RAMOS). As a result, OTR proofed to be a sensitive indicator for plant vitality. The culture characterization under day/night light conditions, however, revealed a complex interaction between oxygen production and consumption, rendering OTR measurement an unsuitable tool to track plant propagation. However, RAMOS was found to be a useful tool in preliminary studies for process development of photosynthetically active plant in vitro cultures.

Keywords Duckweed · Plant in vitro culture · Process development · Respiration Activity Monitoring System · *Wolffia australiana*

H. Rechmann · M. Biselli
Division Juelich, Universtity of Applied Science Aachen, Ginsterweg 1, 52428 Juelich, Germany

A. Friedrich · D. Forouzan · H. Schnabl · R. Boehm (✉)
Institute of Plant Molecular Physiology and Biotechnology, University of Bonn, Karlrobert Kreitenstr. 13, 53115 Bonn, Germany
e-mail: r.boehm@uni-bonn.de

S. Barth
Department of Pharmaceutical Product Development, Fraunhofer-Institute of Molecular Biology and Applied Ecology (IME), Forckenbeckstraße 6, 52074 Aachen, Germany

Present Address:
H. Rechmann
HiTec Zang GmbH, Ebertsraße 30-32, 52134 Herzogenrath, Germany

Introduction

Species of the duckweed genus, *Wolffia* (*Lemnaceae*), belong to the smallest angiosperm species known (Landolt 1986). They show an extreme reduced plant cormus: leaves and stems are replaced by a fusion of both, called a frond. The frond size is between 1 and 2 mm. Beside their use as animal and human food (Appenroth and Augsten 1996), they have a high potential in biotechnological applications (Landolt and Kandeler 1987), including waste water treatment (Fujita et al. 1999; Bergmann et al. 2000) and cultivation in bioreactors (Eichhorn und Augsten 1969; Thompson 1989) e.g. for the production of recombinant proteins. *W. australiana* is the most suitable for in vitro cultivation in terms of growth and robustness among eleven *Wolffia* species (Friedrich 2005).

Establishment of a valuable in vitro production system based on *W. australiana* requires

optimization of culture conditions, medium ingredients and an easy, non-destructive system to monitor plant propagation and vitality of the plants. Cellular gas exchange (O_2 production and consumption) is a suitable parameter since it is highly dependent on the integrity of the cellular processes, representing the overall fitness of the plants.

O_2 exchange optimally can be characterized by the O_2 transfer rate (OTR), i.e. the transfer of O_2 into and out of the medium (Anderlei and Büchs 2000). For easy OTR measurement, the Respiration Activity Monitoring System (RAMOS, HiTec Zang GmbH, Herzogenrath, Germany) was used. It was originally developed for the bioprocess optimization of microorganism cultures (Anderlei et al. 2004). Eight 250 ml Erlenmeyer flasks can be run in parallel under identical conditions. OTR, CTR (CO_2 transfer rate), RQ (respiration quotient), OT (total O_2 transfer) and CT (total CO_2 transfer) are measurable on-line. It already has been used several times on microbial cell cultures (Anderlei and Büchs 2000). RAMOS also was successfully applied to optimize plant suspension cultures of *Azadirachta indica* callus material (Raval et al. 2003), showing the applicability to heterotrophic plant cell suspension cultures. RAMOS, however, never has been used for characterization of a photosynthetis active in vitro culture before.

Therefore, we have attempted to test OTR for in vitro culture characterization of the duckweed species *W. australiana.* OTR was measured under different lighting regimes. Moreover, the feasability to track plant proliferation and vitality was tested.

Materials and methods

Chemicals

Schenk and Hildebrandt medium was purchased from Duchefa (The Netherlands). Ammonium phosphate, sodium nitrate and saccharase were purchased from Merck KGaA (Germany).

Plant material and cultivation

Surface sterile plants of *Wolffia australiana* strain 7211 were cultivated on solidified SH-medium (Schenk and Hildebrandt 1971) supplemented with 2% (w/v) sucrose. The pH was adjusted to 6.0. Cultures were kept at 24 ± 1°C under light of approx. 580 μE m^{-2} s^{-1} in day/night rhythm of 12 h light and 12 h dark.

Plant cultivation in RAMOS vessels

RAMOS is shown in Fig. 1 and the principle of its operation essentially is described by Anderlei et al. (2004). Modified 250 ml Erlenmeyer flasks as part of the RAMOS were autoclaved before use and filled with 50 ml of sterile SH-medium containing 2% (w/v) sucrose. For experiments with elevated concentrations of *N*-sources, the SH medium was supplied with 5 g, 12.5 g and 25 g KNO_3/l, and 0.6 g, 1.5 g and 3 g $(NH_4)H_2PO_4$/l, respectively.

Up to 8 flasks were inoculated with approx. 2,000 Wolffia plants each (approx. 4 g fresh weight) and run in parallel. The flasks were gently shaken in the incubator at 40 rpm to allow homogeneous mixing of the medium beneath the plants which are floating on the surface. The temperature of the incubator was maintained at 24 ± 1°C. Light was supplied by two 0.28 m 18 W-daylight tubes Dulux L 12–950 (Osram, Germany) placed in the incubator above the shaking flasks. This resulted in a photosynthetically active light intensity of approx. 580 μE m^{-2} s^{-1} which was measured with a QSL-2100 quantum meter (Biospherical Instruments, San Diego, CA USA). Light was applied in day/night rhythm of 12 h light and 12 h dark. For specific experiments, plant cultures were exposed to a 16 h/8 h dark/light rhythm or continuous light under otherwise identical conditions.

Measurement of oxygen transfer rate (OTR)

The measuring of OTR was performed with RAMOS (Fig. 1, HiTec Zang GmbH, Herzogenrath, Germany) as described in Anderlei et al. (2004).

Vitality measurement using PAM-2000

As a control, the vitality of the plants was measured with a pulse amplitude measurement

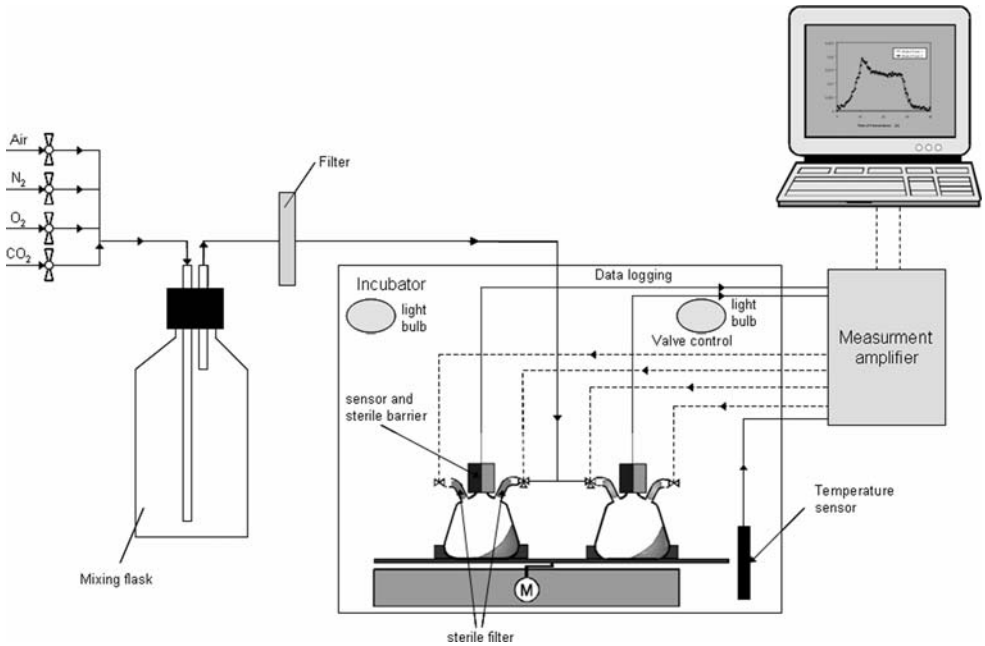

Fig. 1 Principle of Respiration Activity Monitoring System (RAMOS) for characterization of photosynthetically active *Wolffia australiana* in vitro culture. The O_2 transfer rate is detected by measuring the O_2 partial pressure in the gas phase at the top of the flasks in a specific measure phase where all valves are closed and the O_2 partial pressure in the gas phase changes due to cellular oxygen exchange. Each measure phase tested 1 h, following a rinsing phase with fresh air for 2 h. The latter starts with a high-rinsing phase for about 4 min at 100 ml/min, then at 10 ml/min for the rest of the rinsing phase. This measuring and rinsing cycle was continuously repeated. The slope of the O_2 partial pressure is monitored by an electro-chemical sensor (galvanic cell, lead-oxygen battery)

system (PAM 2000, Heinz Walz GmbH, Effeltrich, Germany).

Sucrose determination

To monitor the uptake of sucrose from the medium, the concentration of sucrose was determined enzymatically-amperometically with a glucose-analysator (EBIO Compact, Eppendorf, Germany). The sucrose was enzymatically split into glucose and fructose by saccharase.

Results and discussion

Only few reports are available to date dealing with the use of OTR to characterize and optimize plant in vitro cultures. These are limited to heterotrophic plant cell suspension cultures, e.g. of *Vitis vinifera* (Pepin et al. 1995) and *Catharanthus roseus* (Bond et al. 1988). The aim of the present study was to test the feasibility of OTR measurements to characterize important culture parameters of the photosynthetic active in vitro culture of duckweed (*W. australiana*) like growth or vitality. OTR easily was measured by RAMOS (Anderlei and Büchs 2000) in 250 ml Erlenmeyer flask equivalents (Fig. 1).

Characterization of OTR during plant cultivation under different lighting regimes

In a first set of experiments, RAMOS was used to characterize the OTR in dependency of light conditions. Dark/light-rhythms of 12 h/12 h and 16 h/8 h, and continuous light were applied to *Wolffia* cultures and the OTR was monitored over 72 h (Fig. 2). In the dark, O_2 is consumed by respiration in the mitochondria, which results in positive OTR values of about 1.0×10^{-4} mol l^{-1} h^{-1}. In contrast, in light, oxygen was produced by assimilation but consumed at the same time by

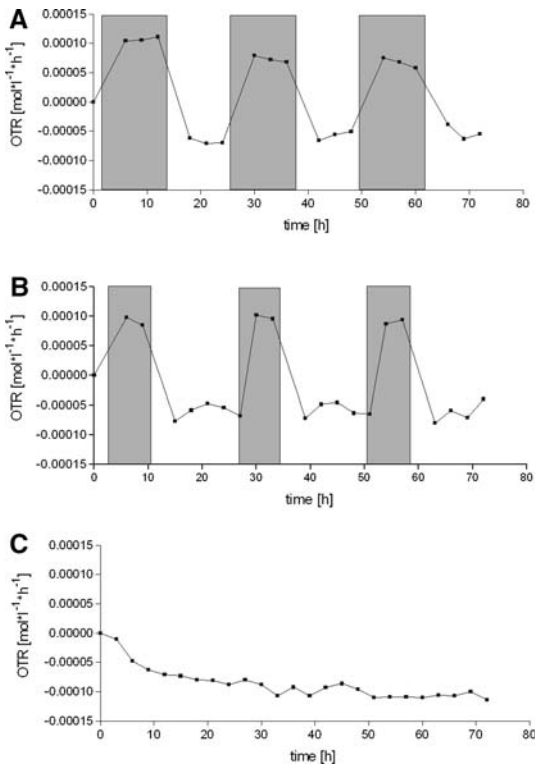

Fig. 2 Influence of light regime on O_2 transfer rate of *W. australiana* cultures. Flasks containing 50 ml of SH medium + 2% (w/v) sucrose were inoculated with approx. 2,000 fronds and cultivated (**A**) 12 h/12 h dark/light regime, (**B**) 8 h/16 h dark/light regime, (**C**) continuous light for 72 h on RAMOS. Shadowed boxes indicate dark periods. Typical data are shown exemplarily from one flask

photorespiration. The sum of both processes can be measured as apparent photosynthesis. Because assimilation (oxygen production) over-compensated photorespiration (oxygen consumption), the apparent photosynthesis resulted in negative OTR value of 0.5×10^{-4} mol l^{-1} h^{-1}. This was irrespective of the length of dark and light phases (Fig. 2A, B). These OTR measurements revealed that, in summary, the *Wolffia* cultures consume more oxygen than they produce under dark/light regimes.

Interestingly, respiration and assimilation periods strictly followed the length of dark and light periods of the different lighting regimes (Fig. 2A, B). This indicates a flexible adoption of the plants to altered lighting conditions.

The culture under continuous light constantly showed an apparent photosynthesis twice as high as under dark/light regimes (Fig. 2C) what might be an indication for increased assimilation under continuous light.

Characterization of duckweed cultures by OTR measurement

To characterize the plant culture in more detail over a longer period, 4 flasks were inoculated and plants were cultivated over 400 h under 16 h/8 h dark/light regime with a parallel determination of fresh weight, OTR and sucrose concentration in the medium (Fig. 3). To analyse the effect of sucrose, another 4 flasks were run in parallel under identical conditions, but omitting sucrose in the medium. The fronds started to propagate immediately after inoculation and the plants on sucrose reached the stationary phase after approx 7 days, resulting in doubling their biomass (Fig. 3A). This growth rate is in accordance with previous observation on *Wolffia* cultures in Petri dishes (Friedrich 2005). Sucrose concentration decreased continuously to approx. 60% over the culture period (Fig. 3A). This indicates that the plants take up sucrose as an alternative carbon source besides assimilation, resulting in a mixotrophic way of nutrition of this otherwise autotrophic plant. This probably leads to a reduced apparent photosynthesis, which might explain the above findings that the plants consume more oxygen than they produce (Fig. 2A and B).

The apparent photosynthesis in light periods showed no alteration over the culture period (Fig. 3B, C, dotted lines), indicating that assimilation parallels with photorespiration, steadily compensating each other.

Surprisingly, OTR values in the dark phases similarly did not follow the increase of fresh weight during the cultivation period, but showed a more or less steady course between 2 and 3×10^{-4} mol l^{-1} h^{-1}. Theoretically, the OTR values in the dark should be solely dependent on respiration and, thus, should increase with advanced plant propagation. This observation might be explained by the assumption that the OTR in dark phase is not only due to respiration, but also is influenced by another, O_2-producing process that partially compensates for the O_2-consumption. When both processes are paralleling

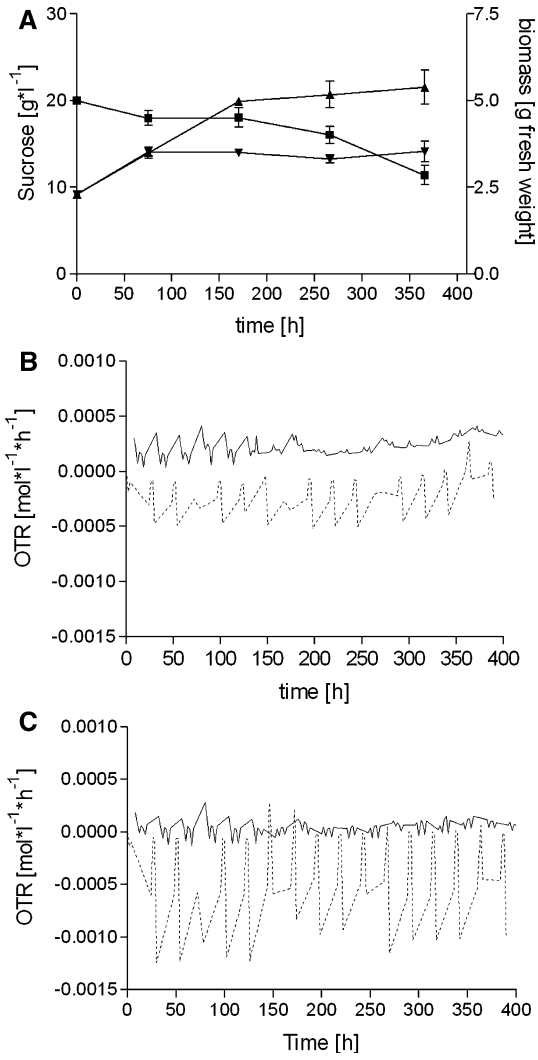

Fig. 3 Culture characteristics of *W. australiana* cultures in RAMOS flasks. Approx. 2,000 fronds per flask were inoculated in 50 ml SH medium with or without sucrose. (**A**) sucrose consumption (■) and frond propagation in medium with sucrose (▲) and without sucrose (▼). (**B** and **C**) OTR values in dark phases (solid lines) and light phases (dotted lines) in medium with sucrose (**B**) and without sucrose (**C**). Data were recorded over approx. 16 days under 16 h/8 h dark/light regime. Values are mean of 4 replicates. The deviation from mean was <20%

with frond propagation, the net OTR value keeps constant, comparably to the partial compensation in the light phases. The nature of an O_2-producing process in the dark phase, however, remains speculative.

These findings are in contrast to the OTR time course in heterotrophic plant cell suspension cultures (Raval et al. 2000; Pepin et al. 1995; Bond et al. 1988), reflecting the much higher physiological complexity of an intact, photosynthetically active plant like *W. australiana*.

The assumption of a partial compensation of the OTR value in dark phases might also explain that the net OTR in the *Wolffia* culture is considerably lower than reported from heterotrophic plant cell cultures, which were in a range 8 up to 38×10^{-4} mol l^{-1} h^{-1} (Raval et al. 2000; Pepin et al. 1995; Bond et al. 1988).

Interestingly, the fronds growing on medium without sucrose showed considerably reduced propagation. The fresh weight only increases by 65% in the first 3 days of culture and remains constant afterwards (Fig. 3A). This may indicates that the plants were cultivated below their optimal photosynthetic capacity due to an insufficient light supply at the surface of the medium. Additionally, the OTR in light phases is decreased up to -1.2×10^{-4} mol l^{-1} h^{-1} and the OTR in the dark phases also decreased to nearly zero, often showing negative values, what is in contrast to the OTR values of the cultures on sucrose containing medium (Fig. 3C). Due to the obvious complexity of the underlying physiological processes, this observation cannot be explained.

Effect of elevated nitrogen concentrations

To test whether the OTR can be used as a sensor for plant vitality, unphysiological high concentrations of nitrogen sources (NO_3^-, NH_4^+) have been added to the SH medium. Equal amounts of plants were cultivated over 90 h under a 12 h/12 h dark/light regime. The OTR values of dark and light periods were averaged over the complete culture time and plotted independently. Results are shown in Fig. 4. The OTR of dark phases remained comparable over a wide concentration range of$(NH_4)H_2PO_4$, but was decreased to approx. 60% in the presence of 3000 mg $(NH_4)H_2PO_4$/l. In contrast, increasing concentrations of KNO_3 paralleled with a decrease of OTR to finally 43% of the original amount. To verify a reduced plant vitality as reason for the observed decrease, chlorophyll fluorescence, an established plant vitality parameter, was measured in parallel,

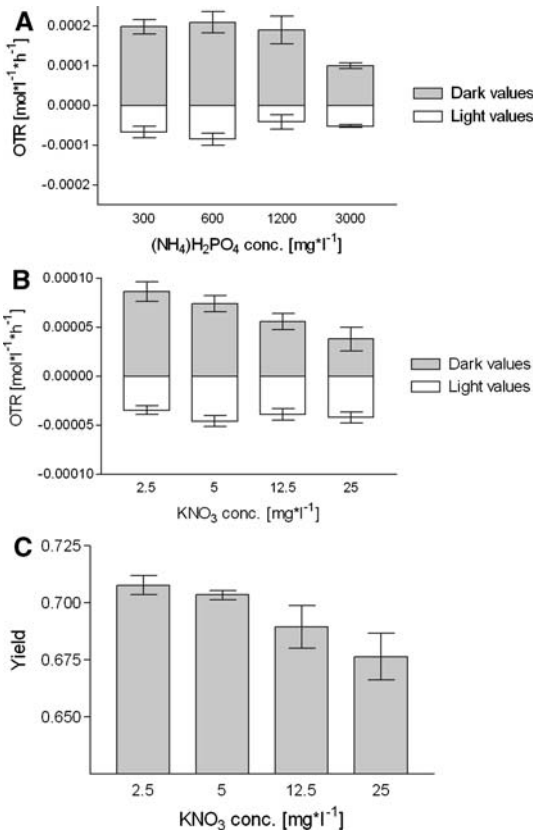

Fig. 4 Influence of elevated levels of nitrogen on OTR of *W. autraliana* cultures. Approx. 2,000 fronds were cultivated over 90 h under a 8 h/16 h dark/light regime on SH media +2% (w/v) sucrose supplemented with enhanced levels of (**A**) $(NH_4)H_2PO_4$ and (**B**) KNO_3. The lowest concentrations represent unmodified SH medium. The OTR of the dark and light periods were plotted independently as mean of all values over the culture period. (**C**) Chlorophyll fluorescence yield in KNO_3-cultures using PAM-2000 fluorometer (see Materials and methods) as an indicator for plant vitality. It records the plant chlorophyll fluorescence before and after a saturating light pulse as a measure for cell vitality (Schreiber et al. 1994). The measuring light was generated by a 655 nm LED in the form of 3 μs pulses at frequencies of 20 kHz. Actinic light sources were supplied by a halogen lamp (white light), a 655 nm LED array and a 735 nm LED. Data are mean of all values over the culture period. Each condition was repeated four times and error bars indicate standard error

using the PAM 2000 fluorescence measuring device (Schreiber et al. 1994) over the whole culture period (Fig. 4C). Here, a similar reduction of plant vitality with increasing concentrations of KNO_3 could be observed. The reduction, however, was not very pronounced. Even on 25 mg KNO_3/l, the vitality was reduced to only 95% of the unmodified medium. Thus, OTR measurement obviously offers a more sensitive tool for vitality determinations than the established vitality assay using chlorophyll fluorescence, especially for the monitoring of a bioreactor culture.

In contrast, the OTR of the light phases remained unchanged under all salt concentration applied. This shows that the apparent photosynthesis is not suitable as an indicator for plant vitality.

Conclusion

The data obtained in this study indicate that respiration in dark culture phases is a suitable parameter to monitor plant vitality of photosynthetically active plant in vitro culture of duckweed (*W. australiana*). This easily can be determined on-line by measuring the OTR in the gas phase of the culture. As a possible application, it can be used for on-line monitoring of plant vitality in bioreactor cultures of *Wolffia* plants.

Additionally, the RAMOS analyses revealed some interesting physiological behaviour of the plants in the in vitro culture. Evidence for a mixotrophic nutrition of *W. australiana* was found when cultured on sucrose-containing medium, resulting in increased propagation rate, reduced apparent assimilation and enhanced apparent respiration in comparison to medium without sucrose.

However, the RAMOS analyses, on the other hand, showed that the OTR is not a suitable parameter to monitor the plant propagation since OTR does not parallel with plant propagation. For this, other parameters like e.g. ethylene production, have to be tested.

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