A System for Measuring the In Situ CO₂ Exchange Rates of In Vitro Plantlets

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Additional index words. *Ipomoea batatas*, *Lycopersicon esculentum*, modeling, net photosynthetic rate

Abstract. A system was designed for measuring the CO₂ exchange rates [net photosynthetic rate (P_n) and dark respiration rate] of in vitro plantlets in situ (in the vessel with natural ventilation). The system, excluding gas cylinders, was placed in a growth chamber so that the desired photosynthetic photon flux (PPF) and temperature could be maintained during the measurement. The CO₂ concentration inside the culture vessel (C_i) was indirectly controlled by controlling the CO₂ concentration outside the vessel (C_o). The P_n of the plantlets was estimated based on the measured C_i and C_o at steady state using a gas chromatograph according to the method described by Fujiwara et al. (1987). The performance of the system was demonstrated by measuring the in situ P_n of sweetpotato [*Ipomoea batatas* (L.) Lam., cv. Beniazuma] and tomato (*Lycopersicon esculentum* Mill., cv. Hana Queen) plantlets in vitro under a range of CO₂ concentrations and PPF. The photosynthetic parameters of the P_n model (Niu and Kozai, 1997) for the plantlets were then estimated based on the measured P_n . The preliminary measurements demonstrated the potential application of the system.

The photosynthetic ability of in vitro plantlets sometimes has been considered insufficient to provide a positive carbon balance; therefore, the plantlets have been thought to require sugar as a carbon and energy source. However, recent research had shown that the photosynthetic apparatus of in vitro plantlets is often sufficiently developed (Pospisilova et al., 1996). The low P_n of in vitro plantlets is attributed to the low CO₂ concentration in the culture vessel (C_i) and low PPF during the photoperiod (Jeong et al., 1995; Pospisilova et al., 1996). The P_n of in vitro plantlets is affected by physical environmental conditions (C_i , PPF, temperature, etc.), sugar concentration in the culture medium, growth stage, and other factors (Desjardins et al., 1995). The effects of the foregoing factors on the P_n of in vitro plantlets in situ (in the vessel with natural ventilation) must be fully investigated to optimize the growth of the plantlets.

The P_n of in vitro plantlets has been measured primarily by using a small assimilation chamber with a forced-air mixing system under controlled environments. The CO₂ concentration of the air moving into and out of the chamber is measured using an infrared CO₂ gas analyzer (IRGA) (De et al., 1993; Donnelly and Vidaver, 1984; Grout and Aston, 1978). The physical (e.g., gas diffusion coefficient adjacent to the tissue) and physiological con-

ditions (e.g., water status of the tissue) in the chamber may differ from those found in the culture vessels. The in situ P_n may then differ from that in the assimilation chamber with identical C_i , PPF, and temperature. If in situ P_n is to be investigated, measurement should be conducted in situ.

Several investigators have measured the P_n of in vitro plantlets. Falque et al. (1991) developed a system for the measurement of CO₂ exchange rates of test tube-grown *Vitis rupestris* L. (cv. du Lot) plantlets using an IRGA. A P_n model based on first-order kinetics was presented, and its parameters were estimated according to the measured decrease of C_i in the closed circuit under various PPFs. Fujiwara et al. (1992) monitored the CO₂ concentrations of the inflow and outflow air of the vessels continuously using several IRGAs

throughout the culture period. The CO₂ exchange rates of potato (*Solanum tuberosum* L.) plantlets cultured in three different initial sugar concentrations were then calculated according to the measured CO₂ concentrations on a plantlet basis. The time courses of CO₂ exchange rates of the plantlets were obtained, but photosynthetic parameters could not be determined.

For IRGA, since the flow rate for sampling is at least 0.3 L·min⁻¹, the in vitro environment will be disturbed by the sampling. The above measurements were conducted in vessels with forced ventilation, and thus the measured P_n and its parameters did not reflect the actual values with natural ventilation. The P_n of potato plantlets and explants was much higher with forced ventilation than with natural ventilation (Kubota and Kozai, 1992; Nakayama et al., 1991). The driving force for natural ventilation is the difference in CO₂ concentration between the inside and outside air of the vessel. Use of a filter disc is a common practice in photoautotrophic and/or CO₂-enriched micropropagation.

To determine the in situ P_n , the amount of the air sample should be as small as possible in order not to disturb the in vitro environment. Gas chromatography has the advantages of high accuracy and the requirement for only a small amount (a fraction of a mL) of sample air. However, measurement and control of CO₂ concentration continuously are not possible.

The purpose of this study was to develop a system for the measurement of P_n of in vitro plantlets in situ, i.e., in the vessel with natural ventilation. The in situ P_n of sweetpotato and tomato plantlets was measured to demonstrate the performance of the system. Modeling of P_n was also conducted to show the potential application of the system.

Materials and Methods

Description of the system. The system (Fig. 1) consisted of gas cylinders, flow rate controllers, a gas mixing container, a culture box made from acrylic plates (30 × 20 × 12.5 cm,

Fig. 1. Schematic diagram of the system for measuring the in situ CO₂ exchange rates of in vitro plantlets. Solid lines with arrows indicate the air flow directions; dotted lines with arrows indicate the signal directions. 1: diluted CO₂ gas cylinder; 2: compressed air cylinder; 3: mass flow controller; 4: air flow rate control valve; 5: air mixing container; 6: sampling pipe (0.6 mm in diameter); 7: culture vessel (polycarbonate Magenta type, 370 mL); 8: plastic cup containing plantlet and medium; 9: thermocouple and quantum sensor; 10: culture box (30 × 20 × 12.5 cm); 11: infrared CO₂ analyzer for monitoring the CO₂ concentration in the culture box; 12: recording device; 13: fluorescent lamps.

Received for publication 30 Jan. 1998. Accepted for publication 27 Apr. 1998. This work was supported by a Grant-in-Aid for Scientific Research (No. 97138) from the Ministry of Education, Science and Culture, Japan. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

in which culture vessels were placed), and a recording device. All but the gas cylinders were placed in a growth chamber in order to control the temperature and *PPF* at desired levels during the measurement.

The flow rate from the compressed air cylinder was kept constant (1.0 L·min⁻¹). The desired CO₂ concentration in the culture box was obtained by adjusting the flow rate from the gas cylinder, containing diluted CO₂ (2%) and air, using a thermal mass flow controller.

Three culture vessels (polycarbonate Magenta-type, 370 mL) were placed in the culture box: two for culturing plantlets and one for monitoring the temperature and *PPF* in an empty vessel. The holes (10 mm in diameter, 65 mm above the bottom of the vessel) on each side of the vessel were covered with gas-permeable microporous filter discs (Mill-Seal, Millipore, Tokyo; pore size 0.5 μm). The number of air exchanges of the vessel was estimated to be 6.5 per hour according to the method described by Kozai et al. (1986). A pipe (0.6 mm in diameter) was fixed between the sides of the vessel and the culture box for sampling the gas in the vessel.

The CO₂ concentration in the culture box was monitored using an IRGA (ZFP9; Fuji Electric Co., Tokyo) throughout the measurement period. The temperature and *PPF* in the empty vessel were monitored by using a thermocouple and a quantum sensor, respectively. A recorder was used during the measurement to check whether the CO₂ concentration, temperature, and *PPF* in the culture box reached steady state.

Measurement of CO₂ concentration. When CO₂ concentration reached a steady state under each set of conditions, the air in the culture box and in the vessel was sampled, and the CO₂ in the sampled air was analyzed using a gas chromatograph (GC-12A, Shimadzu Co., Kyoto, Japan). The *P_n* was calculated by using the average of two CO₂ measurements.

Plant materials and culture conditions. Sweetpotato and tomato plantlets were prepared as follows. For sweetpotato plantlets, one single-node leafy cutting was excised and planted aseptically in a plastic cup (25 mm in diameter and 30 mm in height). Four plastic cups, each containing one cutting and 10 mL Murashige and Skoog (1962) agar (8 g·L⁻¹) medium, with (30 g·L⁻¹) or without sugar, were placed in a 370-mL polycarbonate Magenta-type vessel. The number of air exchanges of the vessel was estimated to be 7.1 per hour. For tomato plantlets, one single-node cutting (without leaves) was excised and planted aseptically in a larger plastic cup (33 mm in diameter and 37 mm in height). Two plastic cups, each containing one cutting and 20 mL Murashige and Skoog (1962) agar (8 g·L⁻¹) medium, with (30 g·L⁻¹) or without sugar, were placed in a 480-mL polycarbonate columnar-type vessel. The number of air exchanges of the vessel was estimated to be 4.0 per hour. No growth regulators were added to the medium and the pH of the medium was adjusted to 5.8 before autoclaving. The air temperature in the culture room was maintained at 24–28 °C, *PPF* at 180 μmol·m⁻²·s⁻¹ on the empty shelf

with a photoperiod of 16 h per day, provided by cool-white fluorescent lamps (Model FPL55EX-N; Matsushita Electric Co., Tokyo). The CO₂ concentration in the culture room was maintained at 1400 μmol·mol⁻¹ for sweetpotato and 400 μmol·mol⁻¹ for tomato plantlets. Sweetpotato and tomato plantlets were cultured under the above conditions for 16 and 30 d, respectively, before transferring to the system for CO₂ measurement.

Estimation and modeling of *P_n*. At the steady state, *P_n* was estimated using the following equation (Fujiwara et al., 1987). The diffusivity of CO₂ in the vessel was assumed to be large enough to achieve a uniform spatial distribution of CO₂.

$$P_n = k \cdot N \cdot V \cdot (C_o - C_i) / LA$$

where *k* is a conversion coefficient, *N* is the number of air exchanges of the vessel (6.5 per hour), *V* is the volume of air in the vessel (370 mL), *C_o* is the CO₂ concentration outside the vessel (i.e., in the culture box) in μmol·mol⁻¹, *C_i* is the CO₂ concentration inside the vessel, and *LA* is the leaf area of plantlets per vessel (m²).

The following *P_n* model (Niu and Kozai, 1997) was used to fit the above measured *P_n* for sweetpotato and tomato plantlets, and the parameters (*P_m*, *f_c*, and *f_i*) of the model were estimated according to the least squares method.

$$P_n = P_m \cdot [1 - \exp\{-f_c \cdot (C_i - C_c)\}] \cdot [1 - \exp\{-f_i \cdot (PPF - I_c)\}]$$

where *P_m* is the maximum *P_n* at CO₂ and light saturation points (μmol·m⁻²·s⁻¹), *C_c* is the CO₂ compensation point (μmol·mol⁻¹), *I_c* is the light compensation point (μmol·m⁻²·s⁻¹), *f_c* is the parameter related to the slope of *P_n* vs. *C_i* curves at *C_i* = *C_c* (mol·μmol⁻¹), and *f_i* is the

parameter related to the slope of *P_n* vs. *PPF* curves at *PPF* = *I_c* (m²·s·μmol⁻¹).

The CO₂ concentrations, which were measured at 26 °C for 5 h or longer after sealing the vessels containing plantlets, were assumed to be *C_c* of sweetpotato and tomato plantlets, respectively. A value of 8 μmol·m⁻²·s⁻¹ was given to *I_c* (Nobel et al., 1993) for both species.

Results and Discussion

Measured and simulated *P_n* of sweetpotato plantlets. Regardless of the culture method, *P_n* increased with increasing *C_i* and became saturated at a *C_i* of ≈1500 μmol·mol⁻¹ under the three different *PPF*s (Fig. 2). Increasing the *PPF* under high *C_i* greatly increased *P_n*. Under constant *C_i* and *PPF*, *P_n* was higher for plantlets cultured under the photoautotrophic than that under the photomixotrophic conditions. This indicates that sugar in the medium suppressed the photosynthesis of the plantlets to some extent. The simulated *P_n* vs. *PPF* curves under three different levels of *C_i* (Fig. 3) indicate that, regardless of *C_i*, *P_n* increased with increasing *PPF* when *PPF* was <1000 μmol·m⁻²·s⁻¹. The estimated parameters *P_m* (μmol·m⁻²·s⁻¹), *f_c* (10³ mol·mol⁻¹), and *f_i* (10³ m²·s·mol⁻¹) of the *P_n* model for sweetpotato were 16.4, 3.0, and 2.5, respectively, for photoautotrophically cultured plantlets, and 12.9, 3.6, and 2.0, respectively, for photomixotrophically cultured plantlets.

Measured and simulated *P_n* of tomato plantlets. The *P_n* of tomato plantlets, cultured under photomixotrophic conditions, increased with increasing *C_i* under each *PPF* and became saturated at a *C_i* of ≈1500 μmol·mol⁻¹ (Fig. 2). Increasing the *PPF* significantly increased *P_n* under high *C_i*. The *P_n* approximately doubled when *PPF* was elevated from 80 to 160

Fig. 2. Measured (symbols) and simulated (lines) net photosynthetic rates (*P_n*) of sweetpotato and tomato plantlets as affected by CO₂ concentration in the vessel (*C_i*, μmol·mol⁻¹) and *PPF* (μmol·m⁻²·s⁻¹).

Fig. 3. Simulated *P_n* of sweetpotato and tomato plantlets as affected by *PPF* (μmol·m⁻²·s⁻¹) and CO₂ concentration in the vessel (*C_i*, μmol·mol⁻¹).

$\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at high C_i . The simulated P_n of tomato plantlets increased with increasing PPF under the three different levels of C_i when PPF was $<1000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Fig. 3). The estimated parameters P_m ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), f_c ($10^3 \text{ mol}\cdot\text{mol}^{-1}$), and f_i ($10^3 \text{ m}^2\cdot\text{s}\cdot\text{mol}^{-1}$) of the P_n model were 16.4, 3.0, and 2.5 for tomato plantlets, respectively.

The above measurement was conducted under steady state. Generally, some time is required to reach a steady state after changing the PPF and CO_2 concentration in the culture box. In the present experiment, since the number of air exchanges of the vessels used for measurement was relatively high (6.5 per hour), about 1 h was required to reach the steady state. Dark respiration rate of the plantlets can also be estimated using the same method.

Measurements can also be conducted under nonsteady conditions using the same system. In this case, the time course of C_i after the start of the photoperiod is measured, and photosynthetic parameters and P_n can be estimated by numerical computation as demonstrated by Niu and Kozai (1997). Likewise, the dark respiration rate under nonsteady conditions can be obtained by measuring the increase of C_i after the start of the dark period. This method allows rapid measurement but requires numerical computation to obtain the parameters of a P_n model, and from the model, P_n can be calculated. Similar numerical computation approaches for estimating P_n and dark respiration rate were previously reported (De Riek, 1995; Falque et al., 1991; Jackson et al., 1994).

If the flow rates at the inlet and outlet of the culture box are accurately measured, P_n can be calculated from the flow rates and the difference in CO_2 concentrations of the air between the inlet and outlet of the box, instead of using the equation presented by Fujiwara et al. (1987). In this case, IRGA can be used for measuring the CO_2 concentrations of the air at the inlet and outlet of the box, but C_i at a steady state is determined by using a gas chromatograph as mentioned above.

From the above example and discussion, we conclude that this system can be used to measure the CO_2 exchange rates (P_n and dark respiration rate) of in vitro plantlets in a vessel with natural ventilation. Photosynthetic ability of in vitro plantlets can be compared under various environmental conditions, with varying initial sugar concentrations in the medium, and/or at different stages. This system may contribute to the study of photosynthesis and carbon nutrition of in vitro plantlets.

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