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In Situ Measurement of Microclimate for the Plantlets cultured *In Vitro*

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Plant tissue culture is a very important technique for the development of the orchids industry. In order to enhance the quality of the plantlets, the microclimate in the culture vessel needs to be detected. An *in situ*, real time and continuous measurement system of *in vitro* plant tissue culture is very useful to study the physiological functions and factors affecting quality. In this study, some sensing elements are adapted to measure the variations of air temperature, oxygen (O_2) and carbon dioxide (CO_2) concentrations in the culture vessel. These gas sensors were calibrated by a gas divider. All sensing elements are kept in a clean condition by alcohol wiping and ultra-violet light exposure. The trend of O_2 and CO_2 concentrations all indicate the validity and adequacy of this measuring system. All sensors can be maintained in good condition for the duration of long-term measurements. This technique provides a novel way to study the physiological functions of plants *in vitro*.

1. Introduction

Micropropagation has become an important industry for the agricultural biotechnology. In this technique, plantlets are established *in vitro* in small and aseptic culture vessels. The water and the nutritional requirements are supplied by the culture medium. The special environmental conditions within the culture vessel include high relative humidity, low carbon dioxide (CO_2) concentration in day light equivalent periods and high CO_2 concentration in dark periods (Kozai *et al.*, 1992). The growing conditions require a low density of microorganisms to be maintained and thus the gas exchange rates in tissue culture vessel are limited (Chen & Chen, 2002).

The most important environmental factors affecting growth of plantlets are the air temperature, and relative humidity, light irradiance, and CO_2, oxygen (O_2) and ethylene (C_2H_4) concentrations. Several researchers have reported that high relative humidity in culture vessels induced plantlet physiological disorders (Ghashghaie *et al.*, 1992; Preece & Sutter, 1990). Buddendorf-Joosten and Woltering (1994) found that the limitation of CO_2 concentrations in a light period inhibited growth of plantlets and induced senescence.

The external environment of culture vessels and the physical properties of vessels influence the internal environment of the vessels. The environmental model for the air temperature, relative humidity and light irradiance have been developed and validated (Chen, 2003, 2004, 2005). The physical properties of culture vessels for plant tissue culture have been studied (Huang & Chen, 2005). However, an *in situ*, on-line and continuous measurement system of CO_2 and O_2 has not been reported.

Kozai *et al.* (1995) introduced two methods to observe the variation of CO_2 concentration. The first method was to use syringes to take gas samples from the culture vessels. The sample gas then was injected into the carrier of a gas chromatograph. The CO_2 concentrations were detected by a hot wire thermal conductivity detector or a flame ionisation detector. This technique was an off-line and discontinuous method to detect the microclimate of internal culture vessels. The frequency of sampling increased the susceptibility to contamination. The accuracy in measuring of CO_2 concentration is limited by the performance of the gas chromatograph. Doi *et al.* (1989) had applied this method to study the C_3 species and crassulacean acid metabolism (CAM) plantlets

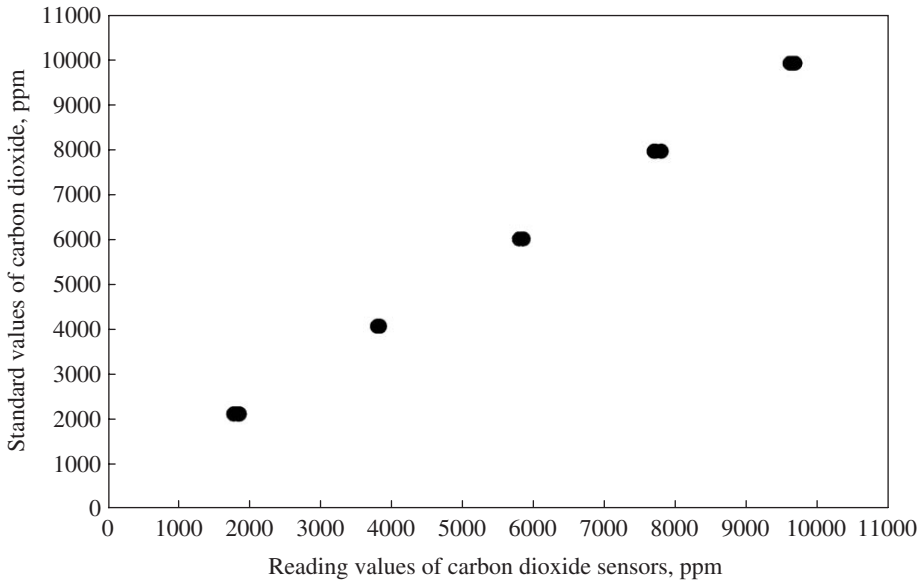

Fig. 1. Relationship between reading values and standard values made by gas divider for carbon dioxide sensor

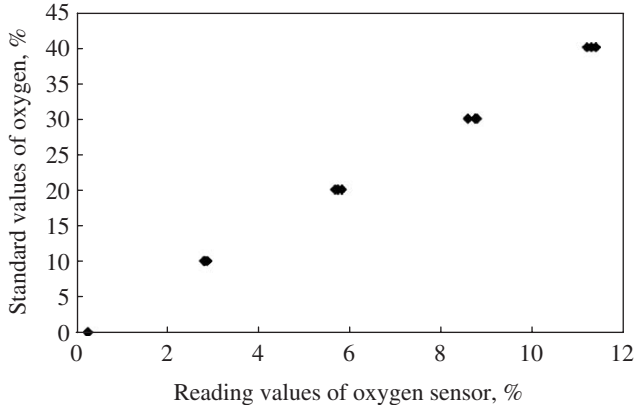

Fig. 2. Relationship between reading values and standard values made by gas divider for oxygen sensor

cultured in vessels and revealed the same measuring problems.

The second method used was a forced ventilation technique (Falque *et al*., 1991; Desjardins *et al*., 1992). Conditioned air was introduced into the culture vessel by the pre-determinated mixing ratio of CO_2 and nitrogen (N_2) gases. The outlet air was sent into the inlet of an infrared gas analysed to continuously monitor the CO_2 concentrations. To avoid the hydration function of medium in culture vessel, inlet air must be humidified with distilled water to maintain the saturated state. The disadvantages of this technique were the complexity of the whole system and the disruption of the growing conditions for the plantlets with force ventilation. In the actual conditions for the plantlets in the culture vessel, the air was nearly stable.

The effects of CO_2 concentration on growth of plantlets *in vitro* have been mentioned by many researchers (Vina *et al*., 1999; Vyas & Parohit, 2003; Desjardins, 1995; Solarova, 1989; Kozai & Sekimoto, 1988). However, the research results were inconsistent. Some measurements were executed by a daily point determination for a long culture period (Righetti *et al*., 1993; Navarro *et al*., 1994). Some CO_2 determinations were done with a number of data during a light-dark cycle only (Doi *et al*., 1989; Hayashi *et al*., 1995). The performance of detecting techniques for gas concentrations may influence the measurements. The information of the physiological function of plantlets *in vitro* in culture vessels is very important to enhance the progress of tissue culture industry. The accuracy of the measurement data for the internal microclimate of the culture vessels can ensure the validity of the basic information on the plantlets. The objective of this study is to develop and validate an *in situ*, real time and continuous measuring technique for O_2 and CO_2 concentrations in the culture vessels.

2. Materials and methods

2.1. *Culture vessels*

The internal microclimate (air temperature, O_2 and CO_2 concentrations) was measured of a conical flask (F-1, I-Shin Co., Taiwan). The design and physical properties of this vessel are described by Chen (2003).

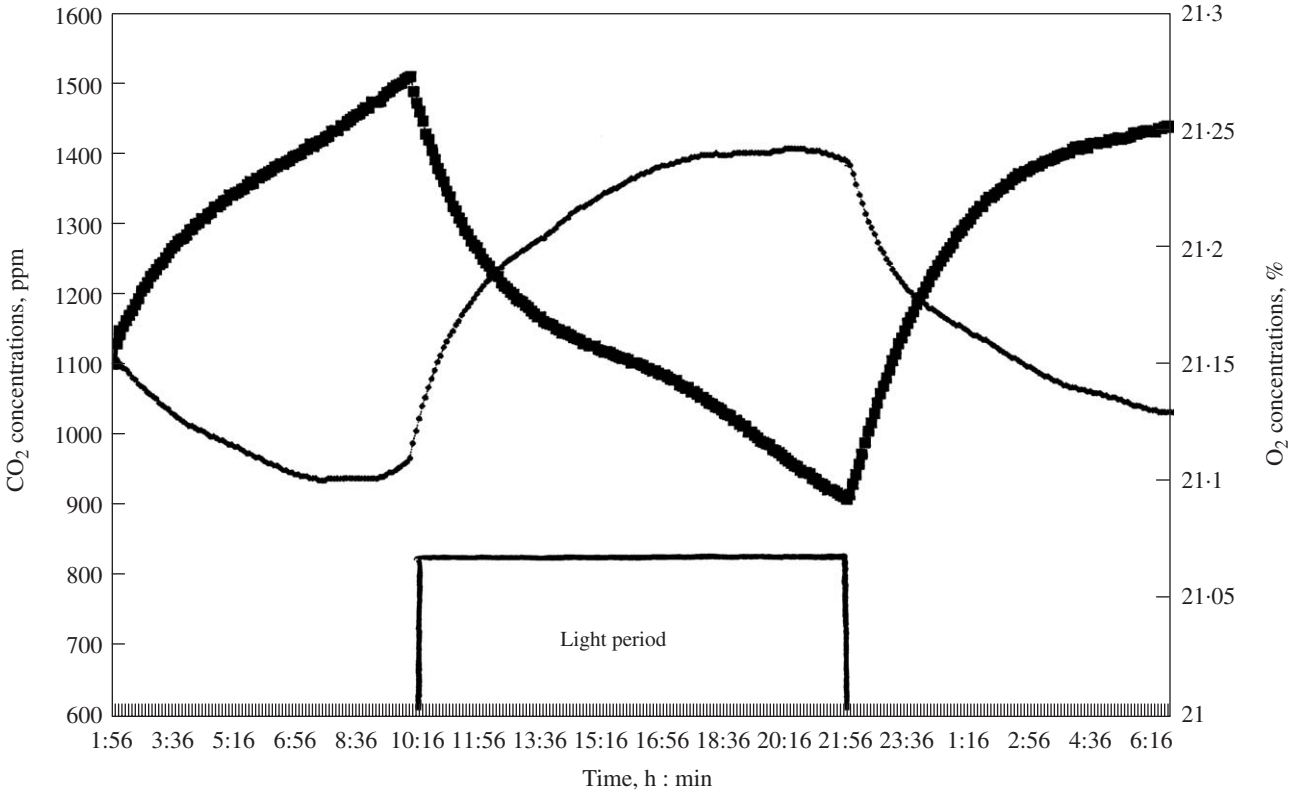

Fig. 3. Carbon dioxide and oxygen concentration distributions over 29 h periods for a conical tissue culture vessel planted with Oncidium plantlets with 30 μmol m^{-2} s^{-1} irradiance: ■, CO_2 concentration; ◆, O_2 concentration

2.2. *Plantlet material*

Two kinds of orchids and one variety of strawberry were used in this study. The usable shoots of *Phalaenopsis* (*P. Taisuco Kochdian* "KH#2") were transplanted on a rooting medium for 20 days. There were 16 plantlets in a flask. At the time of transplanting, each plantlet had three leaves. The shoots of *Oncidium* (*Onc. Grower Ramsey*) were transplanted on the same rooting medium for 28 days. There were 25 plantlets in a flask. There were three or four leaves when the measuring work began. The variety of strawberry Douglas (*Fragaria* × *ananassa Duch*) was transplanted into the same rooting medium for 14 days. Twenty five plantlets were cultured in a flask.

2.3. *Measuring equipment*

The sensing elements for internal microclimate measurements of culture vessels must: (1) be easily placed inside the vessel and without interrupting plantlets growth; (2) be easily sterilised by alcohol wiping and ultraviolet exposure and the sterilisation process must not influence their performance; (3) not affect the conditions of internal microclimate by their sensing function; and (4) maintain its performance for a long time. The sensors used in this study were introduced as follows.

2.3.1. *Temperature sensors*

The type-K thermocouple wires (Omega Engineering, USA) were applied to measure the temperature. The diameter of wires was 0·15 mm. The sensing point was placed in the head space of the vessels and the outside air. These thermocouple wires were calibrated using a temperature calibrator (TC-2000, Instrutek As, Norway). The accuracy of these temperature sensors was within 0·1 °C after calibrating.

2.3.2. *Carbon dioxide sensors*

The Vaisala GMD 20 transmitter (VAISALA, Finland) was used to measure the CO_2 concentrations. The sensing device was composed of the infrad ray source, light filter, gas diffusive membrane, sampling cell and light detector. Measuring scale ranged from 0 to 10 000 ppm. The output ranged from 0 to 1·0 V. The diameter of sampling cell was 15 mm.

To ensure the accuracy of these CO_2 meters, the sensing device was calibrated by a SGD710C gas divider

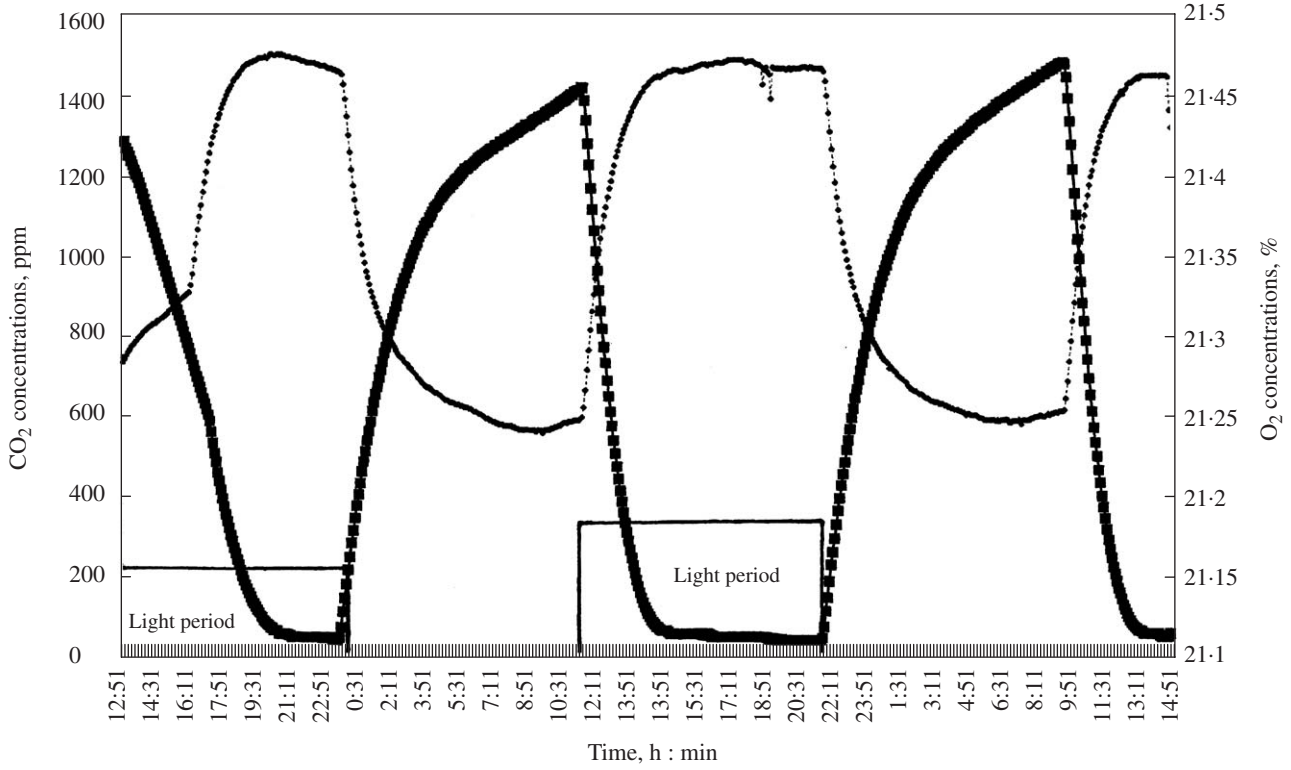

Fig. 4. Carbon dioxide and oxygen concentration distributions for a conical tissue culture vessel planted with Oncidium plantlets at two levels of irradiance, period I was at 45 μmol m^{-2} s^{-1}, period II was at 60 μmol m^{-2} s^{-1}: ■, CO_2 concentration; ◆, O_2 concentration

(STEC Inc., Kyoto, Japan). The gas receivers of this gas divider were connected to pure CO_2 and N_2 gases. The CO_2 concentrations for calibration were changed by adjusting the mixing ratio of the CO_2 and N_2 of gases.

2.3.3. *Oxygen sensors*

The XLT-11-39 O_2 sensor (Analytical Industries Inc., CA, USA) was adopted to detect the O_2 concentrations. The minimum range was 1% O_2 and the maximum range was 100% O_2. The sensing element is a membrane galvanic cell. The output signal range was from 7 to 13 mA.

This sensor was connected to a signal conditioner to convert the current signal into the voltage signal, The output ranged was from 0 to 1·0 V. The diameter and height of this element are 2·5 and 31 mm, respectively.

This O_2 sensor was calibrated by the SGD710C gas divider. Pure O_2 and N_2 gases were connected to the gas receivers of this gas divider. The O_2 concentrations for calibration were produced by changing a mixing ratio of the pure O_2 and N_2 of gases. The calibrating range was from 0 to 40% O_2.

2.3.4. *Irradiance meter*

Irradiance from fluorescence tubes was measured by LI-190SA quantum sensor (Li-COR Co., USA). The accuracy of the meter was ±3% after calibrating.

2.4. *Data logger*

All signals from sensors were connected to a Delta-T2e data logger (Delta-T devices LTP, UK). As the thermocouple wires were connected to data logger, the voltage signals were transformed into temperatures in °C and recorded. The signals of CO_2 and O_2 sensors were recorded directly. The irradiance, and the CO_2 and O_2 concentration measured every minute and the means of five continuous reading values were recorded.

2.5. *Experimental procedures*

A hole was drilled in the rubber stopper through which passed the sampling cell of CO_2 meter, the

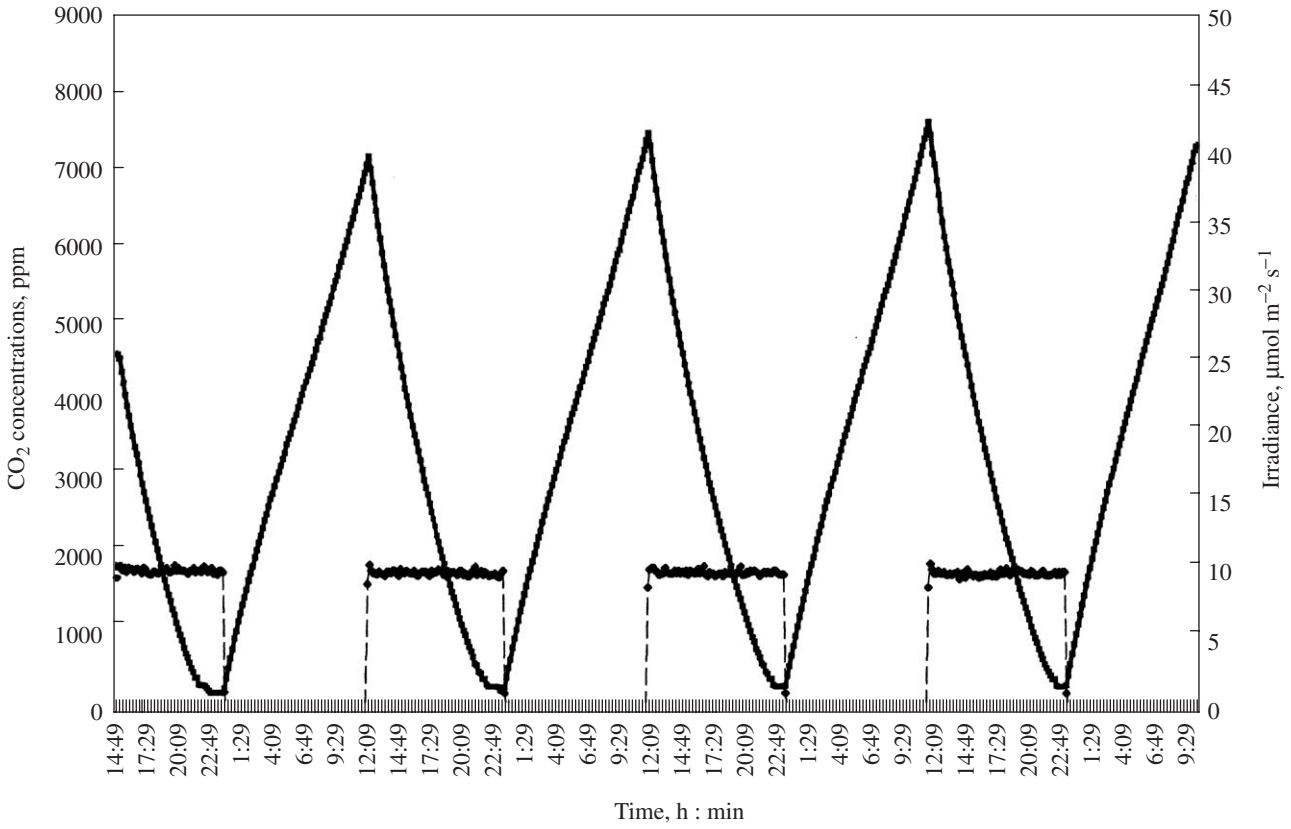

Fig. 5. Carbon dioxide concentration distributions for a conical tissue culture vessel planted with Oncidium plantlets for the long-term test: ■, CO_2 concentration; ◆, light irradiance

connecting wires of O_2 sensor and the thermocouple wires.

Three types of sensors thermocouple wires, O_2 sensing element and CO_2 sampling cell, were placed in the culture vessel. The surface of these elements was treated by wiping with 80% alcohol, placed in the laminar flow cabinet, and then exposed to ultraviolet light for 2 h. The rubber stopper with a hole in central was sterilised in the same way. The vessel lid was removed and these sensing elements were placed to the pre-determined height of headspace of vessel within the laminar flow cabinet. The hole was then sealed using silicon.

The old rubber stopper was removed and replaced with new modified rubber stopper in which sensing elements were set.

After installing all sensing elements, the vessels were removed from the laminar flow cabinet and placed back to the growth chamber.

At the different settings of the external air temperature and irradiance, internal temperature, O_2 and CO_2 concentrations of culture vessel with plantlets were measured and recorded.

3. Results and discussion

3.1. *Calibration equations of carbon dioxide and oxygen sensor*

3.1.1. *Carbon dioxide sensor*

Typical calibration data for the CO_2 transmitter are shown in *Fig. 1*. The quadratic equation was found to be the adequate calibration equation for this transmitter. The calibration equation is as follows.

$$Y_{CO_2} = 278{\cdot}811 + 0{\cdot}950X_1 + 4{\cdot}339 \times 10^{-6}X_1^2 \qquad (1)$$

with a value for determination coefficient R^2 of 0·999, and for standard deviation s of 101·9 where Y_{CO_2} is the corrected value of CO_2 sensor, and X_1 is the reading value of this sensor.

The reading values of CO_2 sensor then was transformed by this calibration equation.

3.1.2. *Oxygen sensors*

Typical calibration data for the O_2 sensor are shown in *Fig. 2*. The adequate model of the calibration

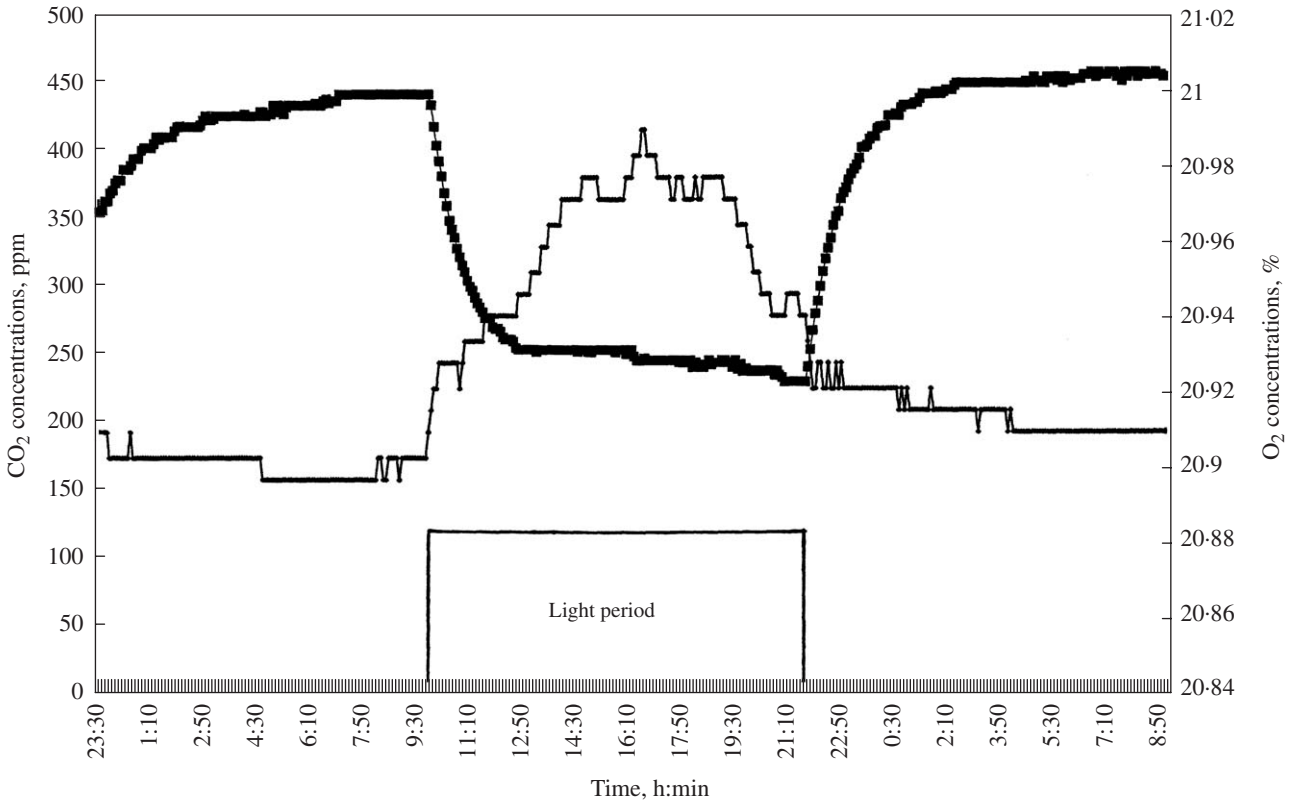

Fig. 6. Carbon dioxide and oxygen concentration distributions for a conical tissue culture vessel planted with strawberry plantlets with irradiance of 50 μmol m^{-2} s^{-1}: ■, CO_2 concentration; ◆, O_2 concentration

equation is

$$Y_{O_2} = -0{\cdot}487 + 3{\cdot}626X_2 - 0{\cdot}003X_2^2 \qquad (2)$$

with a value for R^2 of 0·999, and for s of 0·47; where Y_{O_2} is the corrected value of O_2 sensor and X_2 is the reading value of this O_2 sensor.

3.2. *Distribution of gas concentrations of Oncidium*

A typical distribution of CO_2 and O_2 concentrations in the culture vessel is presented in *Fig. 3*.

In this treatment, the outside air temperature was maintained at 25±1 °C, and the light irradiance was 30 μmol m^{-2} s^{-1}. As the dark period began, the CO_2 concentration began to increase. Due to the dark respiration, CO_2 was released from plantlets and O_2 was consumed, so CO_2 concentration was increased and O_2 concentration was decreased in dark period. This clear pattern of gas concentrations indicated the physiological function of plantlets in dark period.

As the light period began, the concentration of CO_2 decreased and the O_2 concentration increased. The trend of O_2 concentrations could be explained by photosynthesis. The uptake of CO_2 for plantlets by the photosynthesis was more significant than the release of photo-respiration, so net CO_2 concentration decreased.

After the end of light period and the start of dark period, the trend of CO_2 concentrations was increased and the O_2 concentrations were decreased again.

The distribution of CO_2 and O_2 concentrations in the culture vessel for the second treatment was shown in *Fig. 4*. In this treatment, the air temperature was kept at 25±1 °C.

For the light irradiance of 45 μmol m^{-2} s^{-2}, the O_2 concentrations were increased because of the O_2 production by photosynthesis. The CO_2 concentrations were decreased and reached the compensation points after 8 h and 6 min of light period. The required time for cultured plantlets to reach the CO_2 concentrations was 48% of the total light hours. In the compensation point, the CO_2 uptake due to photosynthesis was equal to CO_2 release by photo-respiration.

For the light irradiance of 60 μmol m^{-2} s^{-1}, the increase trend of O_2 concentration was higher than that of lower irradiance of 45 μmol m^{-2} s^{-1}. The depletion of CO_2 concentration under this irradiance was significantly higher than that observed in the treatment of 45 μmol m^{-2} s^{-1}. The period needed for culture plantlets to reach the compensation point was about 4·5 h.

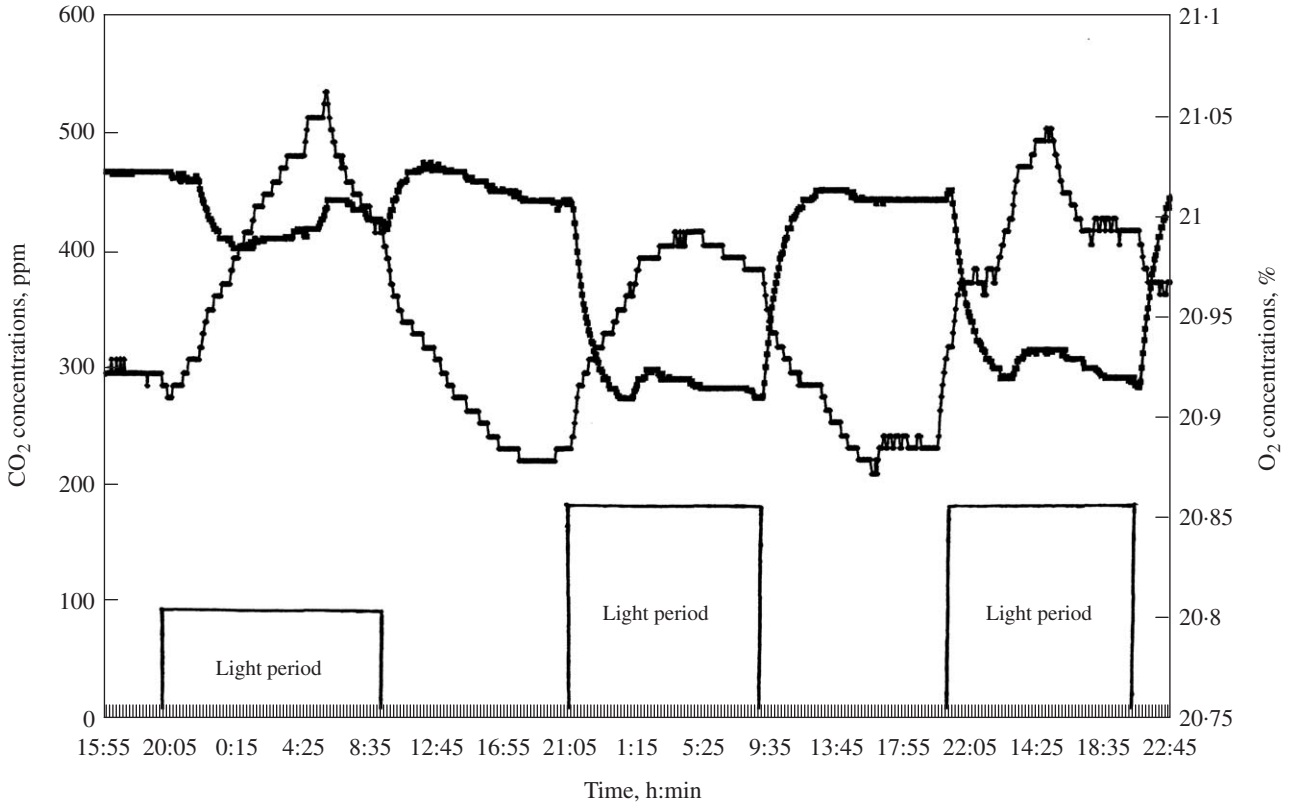

Fig. 7. Carbon dioxide and oxygen concentration distributions for a conical tissue culture vessel planted with strawberry plantlets at three levels of irradiance, period I was at 30 μmol m^{-2} s^{-1}, period II was at 60 μmol m^{-2} s^{-1} and period III was at 70 μmol m^{-2} s^{-1}: ■, CO_2 concentration; ◆, O_2 concentration

The required time to reach this compensation point was nearly 33% of the total light hours. Comparing the two light levels, it was found that the photosynthesis ability of *Oncidium* plantlets was strongly influenced by the light irradiance.

Measurement of internal microclimate in vessels cultivated with *Oncidium* plantlets lasted ten weeks. The results of long term tests with *Oncidium* plantlets treated with 40 μmol m^{-2} s^{-1} are presented in *Fig. 5*. Only CO_2 concentration trends are presented. The fixed variation of the CO_2 trend indicated the stability of the detecting system for measurement of internal microclimate. With the proper sterilisation treatment, no contamination was found in the culture vessel.

3.3. *Distribution of gas concentrations for strawberry*

The typical gas distributions in the culture vessels for strawberry plantlets are shown in *Fig. 6*. The outside environment of this experiment was 25 ± 1 °C and 50 μmol m^{-2} s^{-1}. As the light period start, CO_2 concentration decreased sharply. At the first hour, CO_2 concentration reduced from 450 to 270 ppm. For the next hour, CO_2 concentration reduced gradually to 255 ppm then kept stable for several hours. As the light period was prolonged, the CO_2 concentration decreased slowly.

The O_2 concentration increased as light period began then the trend of O_2 concentration was fluctuated for several hours, and reduced to the lowest level at the front end of light period.

During the dark period, the CO_2 concentration increased rapidly and reached the stable state. The O_2 concentration reduced to a fixed level. Comparing with the trend of gas concentration, the ranges of CO_2 variation of strawberry was significantly smaller than that of *Oncidium*. This result could indicate that the photosynthesis ability of *Oncidium* plantlets is superior to that of strawberry.

The effect of light irradiance on the trend of CO_2 and O_2 concentration of strawberry plantlets is shown in *Fig. 7*. The depletion of CO_2 concentration was significantly affected by the irradiance levels. At the irradiance of 30 μmol m^{-2} s^{-1}, the depletion of CO_2 concentration was varied from 470 to 400 ppm. The depleted range was 70 ppm. The change of CO_2 concentrations was 150 ppm at the 50 μmol m^{-2} s^{-1}

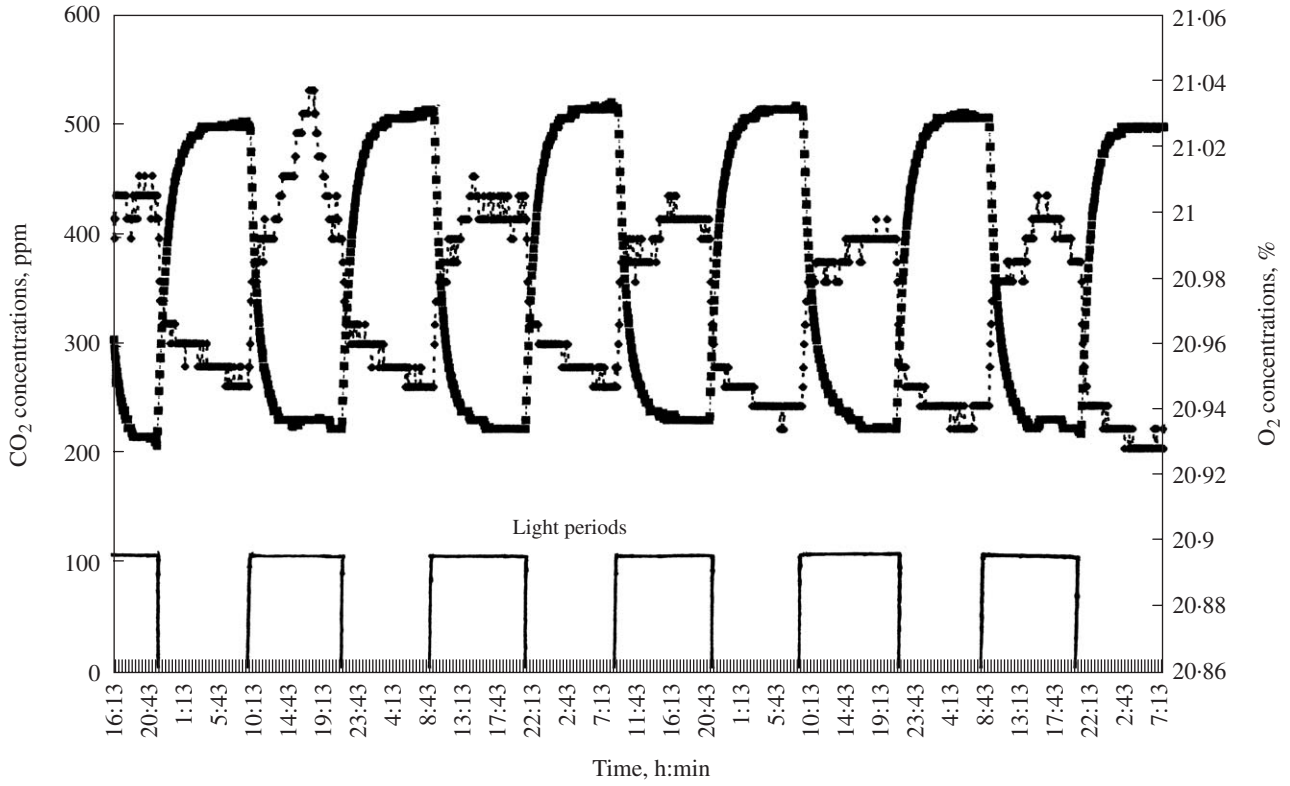

Fig. 8. Carbon dioxide and oxygen concentration distributions for a conical tissue culture vessel planted with strawberry plantlets for the long-term test: ■, CO_2 concentration; ◆, O_2 concentration

level, and 170 ppm at the 60 $\mu mol\,m^{-2}\,s^{-1}$ level, respectively. The required time for CO_2 concentration to reach the compensation point also was influenced by light levels. The required period was 70 min for 30 $\mu mol\,m^{-2}\,s^{-1}$, 60 min for 50 $\mu mol\,m^{-2}\,s^{-1}$ and 48 min for 60 $\mu mol\,m^{-2}\,s^{-1}$, respectively.

The long-term test for this measured system applied for the strawberry plantlets lasted for 6 weeks. The O_2 and CO_2 concentration in the culture vessel during the last week is shown in *Fig. 8*. The fluctuations of CO_2 and O_2 concentrations were stable. The results confirmed the stability of the detecting system.

3.4. *Distribution of gas concentrations for Phalaenopsis*

The trends of O_2 and CO_2 concentration for *Phalaenopsis* plantlets are presented in *Fig. 9*. The outside temperature was $25 \pm 1\,^{\circ}C$ and the light irradiance was kept at 40 $\mu mol\,m^{-2}\,s^{-1}$. The *Phalaenopsis* plants are recognised as a CAM species (Dodd *et al.*, 2002). The path of photosynthesis for the CAM plant could be found in the CO_2 trend of this study. There are four phases for the CAM plant. At phase I (dark period), the stomata of this CAM plant was open and CO_2 concentration was fixed in the cell. The corresponding formation is malic acid. The internal CO_2 concentration of culture vessel was absorbed and the CO_2 level then reached the lowest level.

At phase, the light period started. Photo-respiration produced more CO_2 than the consumption of CO_2 by photosynthesis. The concentration in the culture vessels was increased gradually. The malic acid decarboxylation occurred at phase III, the internal CO_2 concentration of cells released from the malic acid decarboxylation were used for photosynthesis. The CO_2 concentration in the culture vessel was nearly stable. However, this stage is short. As malic acid is exhausted, phase IV starts. Stomata are open and C_3 photosynthesis occurs. The CO_2 in the external air was absorbed. So CO_2 concentration in the vessels was gradually decreased.

From the above discussion, the CAM activity of *Phalaenopsis* plantlets could be found clearly by the measurement of O_2 and CO_2 concentrations.

The effect of temperature on the physiological activity of *Phalaenopsis* plantlets is shown in *Fig. 10*. At the higher temperature (28 °C), the variation of CO_2 concentrations was more obvious than that of lower temperature (23 °C). It indicated that more CO_2 concentrations were absorbed at the higher temperature environment.

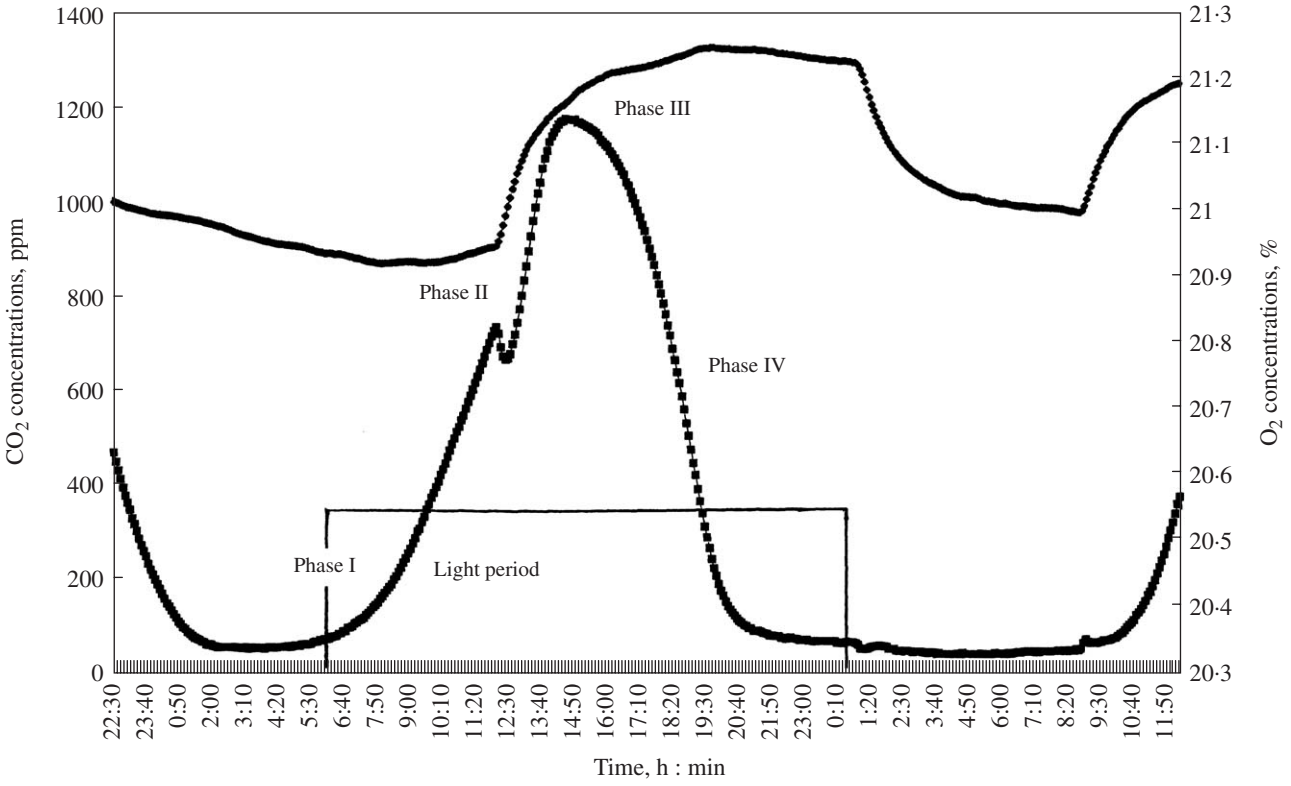

Fig. 9. Carbon dioxide and oxygen concentration distributions for a conical tissue culture vessel planted with Phalaenopsis plantlets with irradiance of 40 μmol m^{-2} s^{-1}: ■, CO_2 concentration; ◆, O_2 concentration

The long-term test of this measuring system for *Phalaenopsis* plantlets was executed for 12 weeks. The trend of CO_2 concentrations in the culture vessels for the data of 11 weeks is presented in *Fig. 11*. The regular distributions of CO_2 concentrations in the culture vessels indicated the long-term performance of this measuring system.

During the growth stage of plantlets in the culture vessels, the internal microclimate influences the physiological function of plantlets. The shoot development, leaf enlargement and root extension are the results of growth of plantlets. Until now, no detail information could be found in literature about the variation of CO_2 and O_2 concentrations in the culture vessel.

From the survey of previous literature, the microclimate data of culture vessels were available only as daily point measurements for a couple days or as interval measuring data over a short time. Many research results were inconsistent. The reason may be attributed to these incomplete data which did not provide enough information for the study of the physiological function. In this study, an *in situ* and online measuring system for detecting O_2 and CO_2 concentrations was developed. Two kinds of orchids with different photosynthesis process and one variety of strawberry with lower photosynthesis ability were used in this study to validate the measuring technique. This system can be applied for long-term measurements. With proper treatment of sterilisation, no contamination was found during the process of microclimate measurement.

4. Conclusions

In this study, a novel detection system was developed to monitor the trend of carbon dioxide (CO_2) and oxygen (O_2) concentration in the culture vessels. The results of the trend of O_2 and CO_2 concentration for plantlets of *Oncidium* and strawberry corresponded to the principle of photosynthesis and respiration process. The function of O_2 and CO_2 concentration for *Phalaenopsis* plantlets showed a clean pattern of crassulacean acid metabolism species. This *in situ* and real time detecting system could be used to measure the variation of O_2 and CO_2 concentrations. These continuously measured data provide useful and detailed information to study the physiological function of plantlets. This system can serve as a tool to enhance the quality of the plantlets.

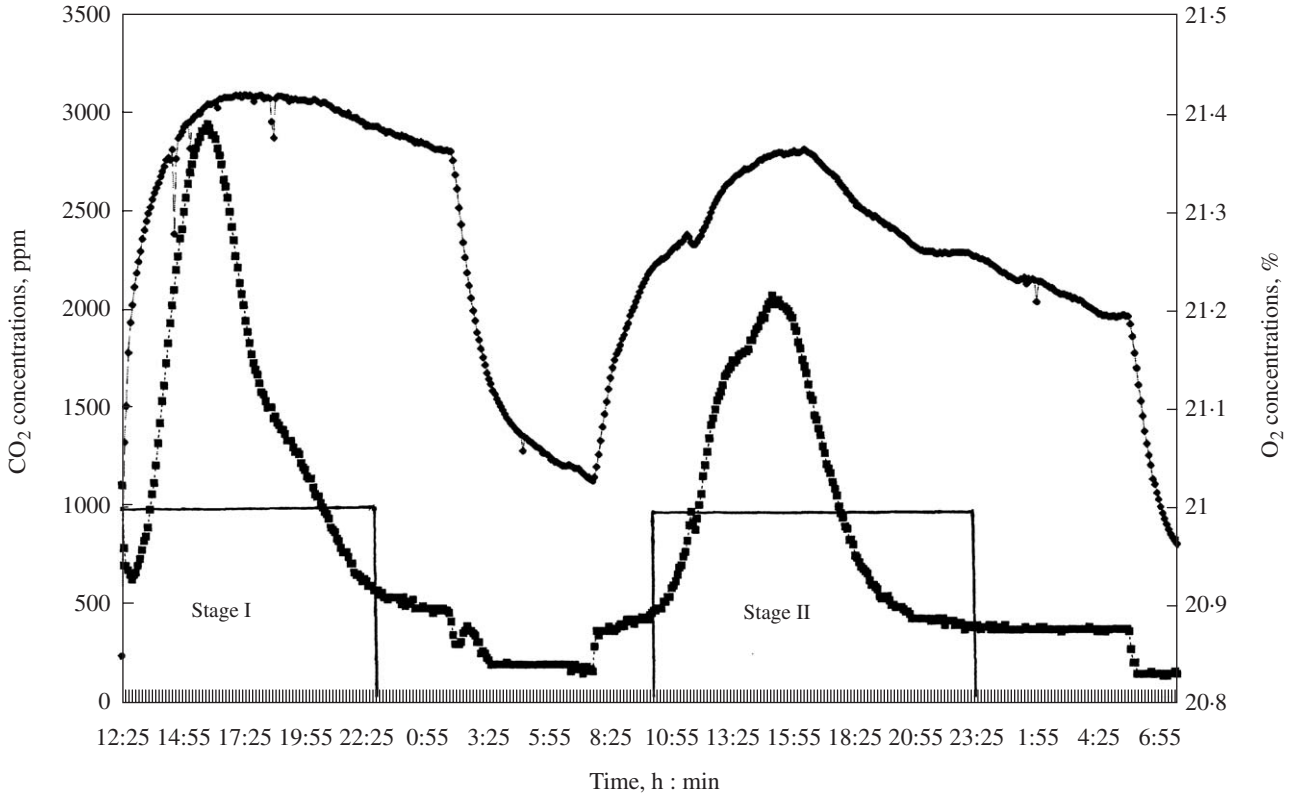

Fig. 10. Carbon dioxide and oxygen concentration distributions for a conical tissue culture vessel planted with Phalaenopsis plantlets at two levels of temperature, stage I was at 28 °C and stage II was at 23 °C: ■, CO_2 concentration; ◆, O_2 concentration

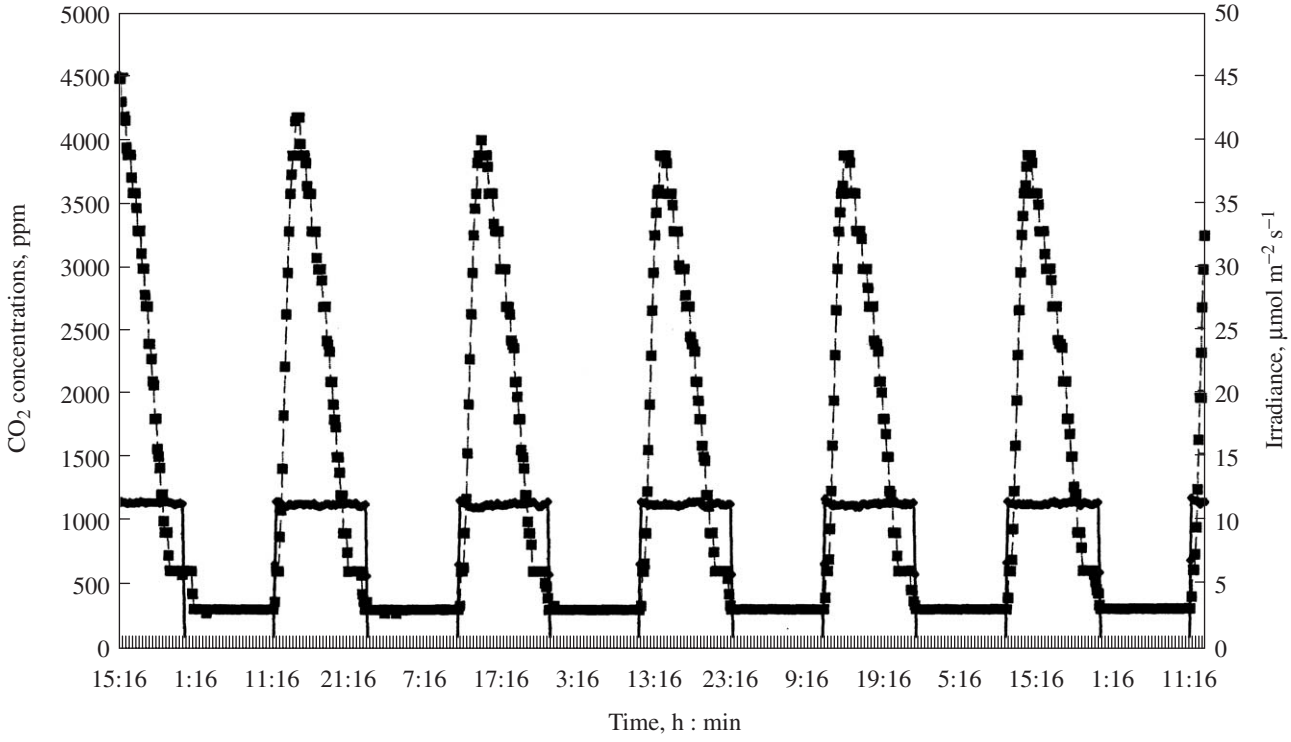

Fig. 11. Carbon dioxide and oxygen concentration distributions for a conical tissue culture vessel planted with Phalaenopsis plantlets for the long-term test: ■, CO_2 concentration; ◆, light irradiance

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